Attorney's Docket No.: 07419-029001 / 00-228

1644 Part of #15

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Gordon et al.
Serial No. : 09/484,577
Filed : January 18, 2000
Title : NUCLEIC ACIDS FOR THE DIAGNOSIS AND TREATMENT OF GIANT CELL ARTERITIS (AMENDED)

Art Unit : 1644
Examiner : Jessica Roark, Ph.D.

Commissioner for Patents
Washington, D.C. 20231

SUBMISSION OF EXHIBITS C-F FOR THE DECLARATION OF LYNN GORDON, M.D. UNDER 37 C.F.R. § 1.132 FILED ON MARCH 20, 2002

Applicants submit herewith Exhibits C-F, which were referred to in the Response and Amendment filed on March 20, 2002.

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EXHIBIT C

Quantitative and genotypic analysis of TT virus infection in Chinese blood donors

Sheng Zhong, Winnie Yeo, Che Kit Lin, Xiao-Rong Lin, Mandy W. Tang, and Philip J. Johnson

BACKGROUND: The TT virus (TTV) is a member of a newly described family of human viruses related to the *Circoviridae* viruses. Its association with specific diseases has not been established, and screening of blood donors has not been implemented. To date, 16 genotypes have been identified.

STUDY DESIGN AND METHODS: Sera from 471 healthy blood donors (aged 11-58 years) were randomly selected and tested for TTV by the use of two sets of primers: NG59d/NG61d/NG63d primers and T801/T935 primers. Quantitative competitive PCR (QC-PCR) was developed to measure the TTV DNA concentration among the blood donors. Sequencing of a part of the genome was performed to identify the various genotypes. Several samples showed a mixed genotype infection.

RESULTS: TTV was detected in 251 (53.3%) of 471 healthy Hong Kong blood donors by the use of NG59d/NG61d/NG63d primers. The prevalence of the virus increased steadily with age ($p = 0.03$). TTV DNA was detected in 90 percent (90 of a randomly selected 100) of samples by the use of T801/T935 primers. TTV DNA concentration was also measured by QC-PCR in the blood donors who were positive for TTV DNA in the first round of the heminested PCR. TTV titers ranged from 4.8×10^2 copies per mL to 6×10^4 copies per mL, with a median value of 1.2×10^4 copies per mL. Sequencing and phylogenetic analysis of a 223-bp fragment from open reading frame 1 showed three main genotypes (G1 [60.7%], G2 [24.3%], and G3 [14%]) and a new genotype 17 (G17), with the latter bearing 60-percent nucleotide homology with other genotypes deposited at GenBank. In addition, a new TTV subtype, G2f, was found.

CONCLUSION: The prevalence of TTV is high in healthy Chinese blood donors. Three main genotypes (G1, G2, and G3) were detected. In addition, a new TTV genotype, tentatively designated as G17, and a new subtype, G2f, were identified.

The TT virus (TTV) is a member of a newly described family of human viruses related to the *Circoviridae* viruses.[1,2] TTV was first identified in the serum of a patient (TT) with posttransfusional non-A-to-G hepatitis by representational differential analysis and was reported to be an unenveloped, single-stranded DNA virus with similarities to the *Parvoviridae* family.[3,4] By the use of heminested primers targeting the N22 region of the TTV open reading frame (ORF-1), TTV sequences were found in more than 45 percent of Japanese patients with fulminant hepatic failure or chronic liver disease, but also in patients with parenteral risk factors (e.g., hemodialysis patients, hemophiliacs, IV drug users) and in 12 percent of blood donors.[4] It was deduced from these observations that TTV may be another candidate virus for cases of unexplained hepatitis. Recent studies from the United Kingdom, Thailand, the United States, Germany, Japan, and South Korea confirmed a high prevalence of TTV infection among the general population, blood donors, and patients with liver disorders, but no clear association could be established between TTV infection and any specific disease.[5-17] The recent detection of a very high prevalence of TTV in Japanese blood donors, by use of another primer pair that targets the untranslated region of the TTV genome (T801/T935), makes even the association between TTV infection and human hepatitis questionable.[18]

ABBREVIATIONS: IS = internal standard; ORF = open reading frame; QC-PCR = quantitative competitive PCR; TTV = TT virus; WT = wild-type.

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Received for publication July 12, 2000; revision received February 9, 2001, and accepted February 23, 2001.

TRANSFUSION 2001;41:1001-1007.

In the initial Japanese study, sequencing a part of the ORF-1 gene showed that TTV could be divided into genotype groups 1 and 2 and then further divided into two subtypes with about 85-percent nucleotide identity (1a and 2a, 2b). Subsequently, genotypes 3 through 11 have been identified.[5,16,17,19-23] The TTV genotypes 12 through 16 were identified in a subsequent publication, which confirmed significant sequence heterogeneity of the genome.[24] The aims of the present study were to assess the prevalence and viral load of TTV in Chinese blood donors and to determine the genotype profiles of the virus strains found in this population.

MATERIALS AND METHODS

Blood samples

Four hundred seventy-one randomly selected serum samples from blood donors were collected at the Hong Kong Blood Bank (Hong Kong Transfusion Service, Hong Kong, China). The samples were centrifuged and the serum stored at -70°C within 4 hours of collection. All samples were tested and found to be negative for HIV and HCV antibodies and for HBsAg by an EIA system (Axsym, Abbott Laboratories, Abbott Park, IL). None of the donors reported a history of liver disease or blood transfusion.

DNA extraction and PCR amplification of TTV sequences

DNA was extracted from 100 µL of plasma by the phenol and chloroform method as described previously, with the addition of yeast tRNA to act as a carrier.[25] Several precautions were taken to avoid contamination, including DNA extraction in a separate room with filtered tips and the use of water as a negative control, throughout the extraction and amplification. Two µL of the extracted material corresponding to a plasma volume of 10 µL was then subjected to seminested PCR for TTV. Degenerate oligonucleotide primers derived from the N22 region, as described by Okamoto et al.,[4] were designed for amplification of most divergent variants currently described to date. This employed the outer primers NG59d, 5′-WCA GAC AGA GGM GAA GGM AAY ATG-3′ (1900-1923) and NG63d, 5′-CTG GCA TYT TWC CRT TTC CAA ART-3′ (2162-2185) in the first round, and the inner primers NG61d, 5′-GGM AAY ATG YTR TGG ATA GAC TGG-3′ (1915-1938) and NG63d in the second round. The reaction mix also contained 1 unit of *Taq* polymerase (Perkin-Elmer AmpliTaq Gold, Roche Molecular Systems, Branchburg, NJ), dNTP (0.2 mM of each nucleotide [Pharmacia, Uppsala, Sweden]), Tris-HCl (20 m*M*, pH 8.4), $MgCl_2$ (3.0 m*M*), and KCl (60 m*M*) in a volume of 20 µL. Amplification was done in a thermocycler (Model 9700, Perkin-Elmer, Foster City, CA) under the following conditions: after denaturation for 9 minutes at 95°C and 40 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 45 seconds. The second round of PCR was performed with 2 µL of the product of the first round with the inner primers for 25 cycles under the same conditions. The viral DNA was also amplified by using T801/T935 primers under the same conditions, but with a total of 55 cycles of amplification. For both PCR assays, amplified products were subsequently identified on a 2 percent agarose gel stained with 0.1-percent ethidium bromide and photographed.

Quantitative analysis of serum TTV DNA concentration

Generation of wild-type and internal standard constructs for TTV. The second round of PCR products of TTV (N22 region) were cloned into a vector (pGEM-T, Promega, Shanghai, China): two to four clones were purified with a kit (DNA Kit, Qiagen, Hilden, Germany), and the products were sequenced. The sequencing reactions for the cloned TTV N22 region were performed in both directions with the M13 forward and reverse primer set using a sequencing reaction kit (ABI Big Dye, Perkin-Elmer) and run on a DNA automatic sequencer kit (ABI 377, Perkin-Elmer). One of those strains, ONCO-17, has three *Rsa*I cleavage sites and was chosen for constructing the internal standard (IS). ONCO-17 was digested by *Rsa*I and subsequently used for transforming *Escherichia coli* DH5α. The resultant clones were subjected to PCR screening using NG61d and NG63d primers. As expected, one of the clones with 156 bp was obtained and was designated as ONCO-17.1. *E. coli* DH5α was transformed with a vector (pEGMONCO-17.1, Qiagen) and the plasmid was purified with a DNA kit (MiniPrep, Qiagen) according to the manufacturer's instructions. Sequencing revealed that an 115-bp DNA fragment in the middle part of the clone is deleted and that corresponding regions of NG61d and NG63d primers remain the same. This deletion leads to 156-bp (rather than 271-bp) PCR products when NG61d and NG63d primers were used to amplify the N22 region of TTV DNA. ONCO-17 and ONCO-17.1 had been used as wild-type (WT) and IS, respectively, for the quantitative competitive (QC-PCR). Plasmid concentrations of the ONCO-17 and ONCO-17.1 were determined by a DNA/RNA calculator (Genequant, Pharmacia) that measured the absorption at 260 nm.

Quantitative determination of TTV by QC-PCR by using IS constructs. For each assay, six tubes, containing 25,000, 5,000, 1,000, 200, 40, or 8 copies of the viral N22 region competitor and 2 µL of DNA (equivalent to 10 µL serum), were subjected to QC-PCR amplification. Competitive PCR for TTV was performed with PCR mixtures consisting of 50 m*M* KCl (pH 8.3), 200 µM of each dNTP, 3.0 m*M* $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase (Perkin-Elmer), 0.2 mM each of NG61d and NG63d, 1 µL of the sample, and 1 µL of the TTV IS construct of known concentration. PCR amplification was performed under standard conditions using the following cycles: 95°C for 10 minutes and then 45 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 50 seconds, and extension at 72°C for 50 seconds,

with a final extension step at 72°C for 5 minutes. PCR products were analyzed on 2-percent agarose gel containing 0.5× Tris-borate-EDTA electrophoresis buffer and 0.5 µg of ethidium bromide per mL. Gel signals were then digitalized and submitted to densitometric analysis. If the sample:competitor denseness ratio equals 271:156, which is the ratio of the expected fragment lengths of the WT and the competitor, then we judge that the sample has the same number of TTVs as the competitor of known concentration.

Gene sequencing and phylogenetic analysis

The amplified products after the second round of PCR were purified from agarose gel using a gel-extraction method (QIAquick, Qiagen). Subsequently, they were cloned into pGEM-T vector (Promega), where two to four clones were sequenced as described above. Multiple sequence alignments were carried out with a software program (Clustal W, version 1.6[26]), and phylogenetic analyses were performed with another software program (Molecular Evolutionary Genetics Analysis [MEGA], version 1.01, available at: www.megasoftware.net). Phylogenetic distances were determined by using the MEGA program, and phylogenetic trees were constructed by the neighbor-joining method.[27]

Statistical analysis

Statistical analysis was performed with statistical software (SPSS-PC, SPSS, Chicago, IL). To compare blood donors with and without TTV infection, we used the chi-square test. We considered p values less than 0.05 to be significant.

RESULTS

Prevalence and quantitative analysis of serum TTV DNA

Of the 471 blood donors aged 11 to 58 years, 251 (53.3%) proved to be positive for TTV by the heminested PCR amplification. The infection rate in males and females was 51.2 percent (127/248) and 55.6 percent (124/223), respectively. The age distribution and the prevalence rates among the blood donors by age groups are shown in Table 1. The frequency of TTV infection increased with age, from 42.2 percent (35/83) in the group aged 17 to 19 years, to 52.9 percent (144/272) in that aged 20 to 29, and 64.4 percent (47/73) in that aged 30 to 39. The peak infection rate of 85.7 percent (6/7) was found in blood donors more than 50 years old. The mean age of the 251 positive and 220 negative persons was 26.9 and 24.8 years, respectively. The results of the first round of testing showed that the distribution of positive rates in different age groups was 6.0 percent (5/83), 21.0 percent (57/272), 26.0 percent (19/73), 30.6 percent (11/36), and 85.7 percent (6/7) in groups aged 17 to 19, 20 to 29, 30 to 39, 40 to 49, and 50 to 59, respectively. Similarly, in the second round of PCR, the frequency of infection increased steadily with age.

TTV DNA was also tested with a second set of primers (T801/T935) in 100 randomly selected samples of the initial 471 samples. TTV DNA was detected in 90 (90%) of these 100 samples (Table 2). Thirty-nine samples were positive in both assays (39/39, or 100% of the NG059d/NG061d/NG063d-positive samples; 39/90, or 43.3% of the T801/T935-positive

TABLE 1. Age-specific distribution of TTV DNA in 471 blood donors*

Age group (years)	Number	TTV PCR-positive: First round (%)	TTV PCR-positive: Second round (%)
17-19	83	5 (6.0)	35 (42.2)
20-29	272	57 (21.0)	144 (52.9)
30-39	73	19 (26.0)	47 (64.4)
40-49	36	11 (30.6)	19 (52.8)
50-59	7	6 (85.7)	6 (85.7)
Total	471	98 (20.8)	251 (53.3)

*$p = 0.030$ (χ^2 test).

TABLE 2. Prevalence of TTV viremia determined by NG059d/ NG061d /NG063d primers (NG primer) and T801/T935 primers in Hong Kong blood donors

	T801/T935-positive	T801/T935-negative	Total
NG primer-positive	39 (39%)	0 (0%)	39 (39%)
NG primer-negative	51 (51%)	10 (10%)	61 (61%)
Total	90 (90%)	10 (10%)	100

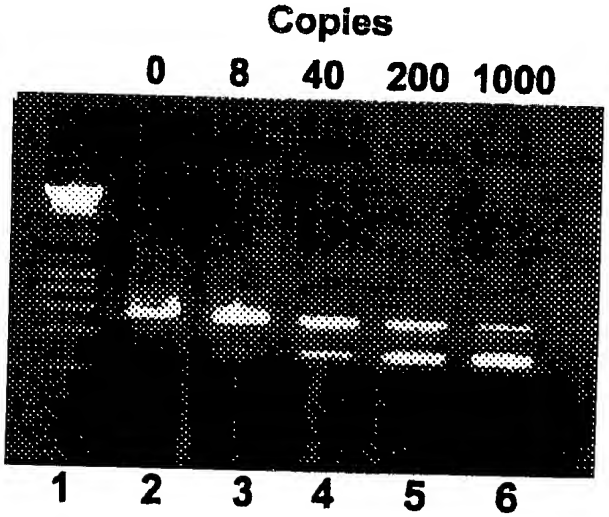

Fig. 1. QC-PCR for TTV. Purified plasmid ONCO-17.1 (IS) containing the deleted fragment was added as a competitor with different copy numbers in each of the PCR. The figure shows an ethidium bromide-stained gel of PCR products after 45 cycles of amplification of the TTV target sequence from a blood donor with a TTV genome burden of 20,000 copies per mL. Lane 1 is a 100-bp ladder. The upper band (271-bp in size) is amplified from the wild-type virus, and the lower band (156-bp in size) is the added competitor. The numbers of copies of added competitor are indicated at the top of each lane.

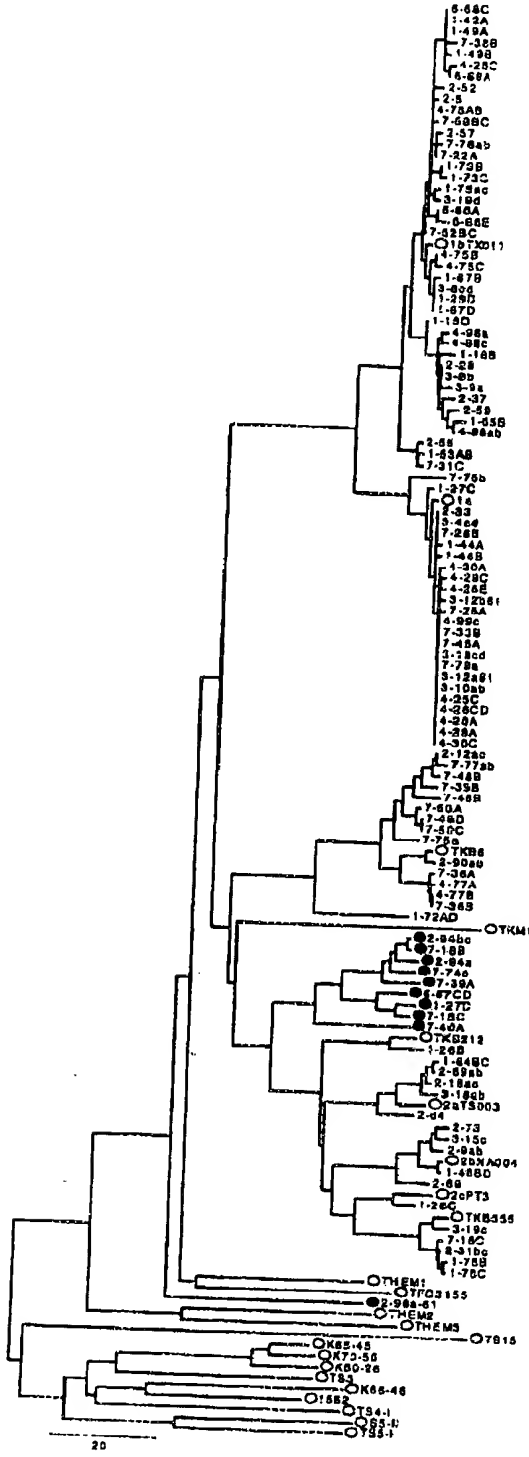

Fig. 2. Phylogenetic analysis of TTV isolates. A phylogenetic tree was constructed for 223-bp fragments from the 107 isolates in this study, and 18 TTV isolates (○; genotypes and isolates also indicated) of genotypes 1 through 16 are reported as references. TTV/17 (g17) and unclassified isolates in Hong Kong, ●.

samples). To quantitate TTV DNA, QC-PCR was used to measure the TTV titer in the 20.8 percent (98/271) of subjects who tested positive in the first round of PCR, on the assumption that they had the highest titer of TTV viremia (Fig. 1). The TTV titer ranged from 4.8×10^2 copies per mL to 6×10^4 copies per mL, with a median of 1.2×10^4 copies per mL.

Phylogenetic analysis of TTV sequences

Figure 2 shows the phylogenetic analysis, on the basis of genetic distance, of TTV sequences derived from 63 randomly selected samples of the 251 TTV-positive blood donors and compares them to published TTV sequences. Results from the sequencing analysis showed that at least four genotypes of TTV were present in Hong Kong. In one blood donor, an unclassified isolate (2-98-61) was found and tentatively designated as genotype 17 (G17), which did not show strong homology to any of the TTV isolates described (Fig. 3). In addition, a group (9 isolates) of TTV isolates close to genotype 2, which could not be classified into any of the subtypes of G2a, 2b, 2c, or 2e, were therefore tentatively designated as G2f. One of the G2f isolates (HK7-40A) was shown to be closely related to a nonhuman isolate (86% homology) (Bo De isolate, Y18912). Several samples showed a mixed genotype infection. By counting of the individual TTV isolates (107 isolates) obtained from the 63 randomly selected blood donors, the distribution of TTV genotypes was shown to be type 1 (G1), 60.7 percent; TTV type 2 (G2), 24.3 percent; TTV type 3 (G3), 14.0 percent; and TTV type 17 (G17), 0.93 percent.

DISCUSSION

Our results show that the prevalence of TTV in Hong Kong is as high as that in blood donors living in Rio de Janeiro, Brazil,[17] and higher than that reported in Japan, South Korea, the United States, and the UK.[4,5,10,14,16] The prevalence of the virus increased steadily with age, and it was found in all groups of donors more than 10 years old. The continuous increase in the prevalence suggests that the virus may not be eliminated and that persistent infection is common. Unfortunately, sequential samples from the subjects tested are not available to confirm this.

In a recent study, using the second pair of primers (T801/T935), TTV DNA was detected in up to 92 percent of healthy Japanese adults[18]; using the same primer pair, we have found similar results. The prevalence rate appears to be significantly different when different primers are applied. All 39 positive cases examined by NG059d/NG061d/NG63d primers were also positive with the second pair of primers (T801/T935), which suggests that the second primer pair targets a more conservative region of TTV strains. Alternatively, it might amplify a variety of TTV-like genomes that share similar sequences in a particular region. In line with this notion, recent studies showed that the higher rate of detection with this primer pair is not related to sensitivity, but rather to speci-

ficity,[24] and that TTV exists as a "swarm" of at least five closely related but different viruses.[25]

We developed a QC-PCR system that allowed accurate and highly sensitive TTV DNA quantitation in the range of 8 to 25,000 copies. This assay corrects both for intertube variations in PCR efficiency and for variability in the amount of input DNA and should, therefore, be useful in quantitating TTV in crude materials. Applying this system, we measured TTV DNA titers ranging from 4.8×10^2 to 6×10^4 copies per mL in a subset of blood donors. The levels of TTV DNA in our blood donors are similar to those reported in a recent Japanese study (3.45 ± 0.67 log copies/mL) by real-time PCR.[29]

It has been suggested that TTV could play a role in the development of fulminant hepatitis,[4,6] and one cannot rule out the possibility that virulent strains of TTV may exist. However, in studies involving the inoculation of TTV into chimpanzees or rhesus monkeys, hepatitis was not induced.[1,30]

Sequencing and phylogenetic analysis of a 223-bp fragment from ORF-1 showed genotypes 1, 2, and 3 and a new genotype, G17, with the latter having low homology with other genotypes deposited at GenBank. We also found unclassified subtypes of TTV in our population, according to the taxonomy suggested by Okamoto et al.[24] It is interesting that one of the isolates (7-40a) showed 86-percent homology with the closest published nonhuman TTV strains.[31] It is thus clear that TTV represents an extremely heterogeneous population and that more genotypes or subtypes may be revealed by further studies. It is also of interest that only four genotypes of TTV—G1, G2, G3, and G17—were found in Hong Kong, as compared to 16 genotypes found in Japan, and this may partly be due to limitation of the present study, which analyzed only about 25 percent (63/271) of the donors who were positive for TTV on the basis of a short fragment of N22.

The prevalence of TTV as measured by the heminested PCR, however, proved to be much higher in Hong Kong. It is also noteworthy that some of the same genotypes are found in Japan, the United States, Thailand, France, Scotland, and Iceland, and Mushawar et al.[1] found no correlation between genotype and geographic origin. Nevertheless, our genotypic analysis of TTV in healthy blood donors gives a general picture of each genotypic distribution of TTV in our population, which may contribute to identifying specific genotypes in association with specific diseases.

Current evidence indicates that the virus can be transmitted by the parenteral route and that it is more common in persons who have received blood transfusions or blood components or who are IV drug abusers,[4,6,8] but the high rate of viremia in our healthy population suggests that transmission is more likely to occur by nonparenteral routes. Mixed infection is common in our blood donors, who have no transfusion history. The high prevalence of TTV viremia in teenagers who have had no blood transfusion history certainly suggests multiple routes of transmission, other than the bloodborne route, such as maternal/fetal, perinatal, sexual, and fecal/oral. Such a notion has been supported by evidence that the virus can be detected in feces, bile, breast milk, saliva, and cord blood.[32-37] In our previous study, the TTV titer measured in saliva samples was 100 to 1000 times higher than that in the corresponding serum, which suggests that TTV may be effectively transmitted through saliva drop-

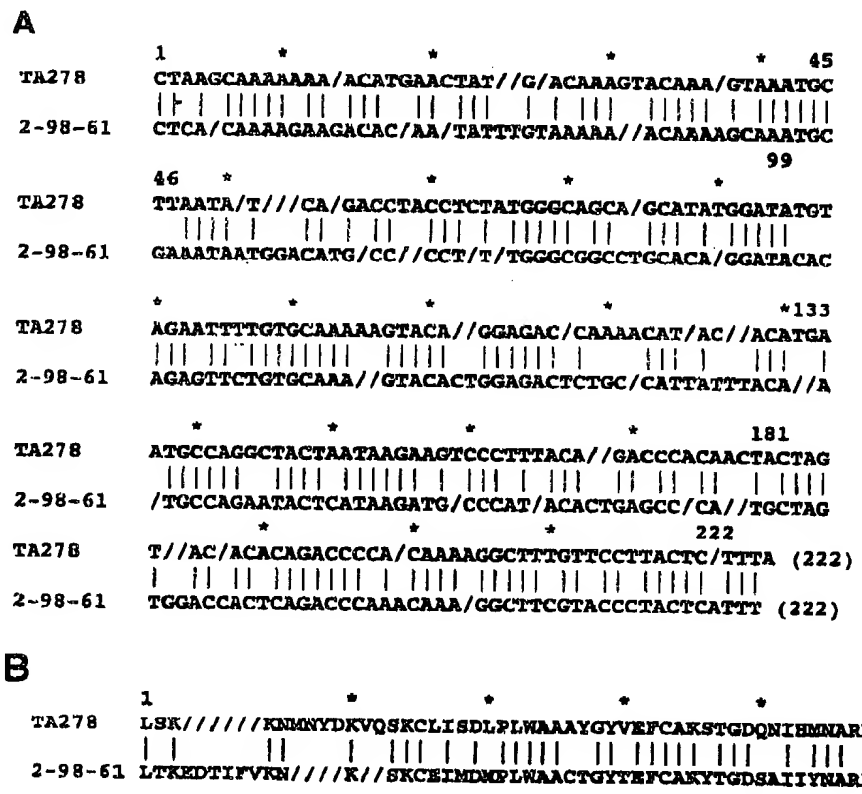

Fig. 3. Nucleotide (A) and amino acid (B) sequences of the new genotype G17 (isolate, 2-98-61). The sequence of TA278 of genotype 1a[4] is indicated at the top. Dashes represent the same nucleotides or amino acids as in the TA278 isolate, and slashes represent deletions of nucleotides or amino acids. The total number of nucleotides or amino acids is indicated in parentheses after the two isolates.

lets.[38] This route of transmission could explain the high degree of exposure to viral infection observed in the general population.

On the basis of our data, we conclude that a very high prevalence of TTV was found in healthy blood donors in Hong Kong. Three main genotypes (G1, G2, and G3) were identified in our population with a frequency of 60.7 percent, 24.3 percent, and 14 percent, respectively. Moreover, a new TTV genotype, tentatively designated as G17, and a new T1V subtype of G2f were found in this population. On the basis of the current findings and reports from other studies, it does not appear that TTV is associated with any specific disease, and it is our opinion that no systemic screening is required for donor blood samples.

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EXHIBIT D

DRUG DISCOVERY
AND GENOMIC TECHNOLOGIES

Research Report

Hybridization Cross-Reactivity within Homologous Gene Families on Glass cDNA Microarrays

BioTechniques 31:1182-1192 (November 2001)

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ABSTRACT

Glass cDNA microarrays can be used to profile the expression of thousands of gene targets in a single experiment. However, the potential for hybridization cross-reactivity needs to be considered when interpreting the results. Here, we describe hybridization experiments with a model array representing four distinct functional classes (families): chemokines, cytochrome P-450 isozymes, G proteins, and proteases. The cDNA clones selected for this array exhibited pairwise sequence identities ranging from 55% to 100%, as determined by a homology scoring algorithm (LALIGN). Targets for microarraying were amplified by PCR and spotted in 4-fold replication for signal averaging. One designated target from each family was further amplified by PCR to incorporate a T7 promoter sequence for the production of synthetic RNA transcripts. These transcripts were used to generate fluorescent hybridization probes by reverse transcription at varying input concentrations. As expected, hybridization signals were highest at the matching target elements. Targets containing less than 80% sequence identity relative to the hybridization probe sequences showed cross-reactivities ranging from 0.6% to 12%. Targets containing greater than 80% identity showed higher cross-reactivities (26%–57%). These cross-reactive signals were analyzed for statistical correlation with the length of sequence overlap, percent sequence identity, and homology score determined by LALIGN. Overall, percent sequence identity was the best predictor of hybridization cross-reactivity. These results provide useful guidelines for interpreting glass cDNA microarray data.

INTRODUCTION

The natural progression of sequencing entire genomes has been to develop parallel hybridization technologies to profile gene expression in biological tissues. These include glass cDNA microarrays that are capable of analyzing the expression of thousands of genes in a single hybridization experiment (3,14, 15,17). In this approach, cDNA clones selected from mRNA expression libraries are amplified by PCR and spotted robotically onto a chemically modified glass surface. Experimental RNA samples are labeled by reverse transcription with the incorporation of fluorescent dyes for hybridization onto the array. Hybridization experiments typically employ a two-color format for the analysis of differential gene expression, for example, between diseased versus normal tissues (5). This technique has been used successfully in human, plant, and microbial gene expression studies (10,11,13). Studies have been reported using large compendiums of microarray expression data for gene target validation and the elucidation of disease-relevant gene pathways (2,9,16).

The purpose of the present study was to establish guidelines for interpreting microarray results where spotted cDNA targets are known or suspected to share moderate to high sequence homology. This problem becomes increasingly important as gene content is expanded toward genome-wide expression analysis. There are numerous examples of functionally related genes sharing high sequence homology within and between genomes (4,6). In addition, an increasing number of alternatively spliced gene transcripts have been identified in the course of sequencing and aligning expressed sequence tags (12), demonstrating that cDNAs derived from mRNA transcripts can also share exact identity over a portion of their sequence.

To our knowledge, no specific cross-hybridization data on microarrays has been published to date. However, hybridization experiments on nylon membranes have shown that cross-reactivity is possible when two different gene targets share 77%–100% sequence identity (19). It has also been stated that cross-reactivity can become significant on glass cDNA microarrays when gene targets share greater than 75% sequence identity (18). Here, we describe a series of cross-hybridization experiments with cDNA sequences selected from four different functional classes and sharing 55%–100% sequence identities.

MATERIALS AND METHODS

Clone Nomination and Homology Scoring

Six gene families were selected for cross-hybridization experiments: chemokines, cytochrome P450 (CYP), G protein gamma subunits, and serine, threonine, and metalloproteases. These genes function primarily in the areas of signal transduction and drug metabolism and have been widely studied as potential drug targets and/or toxicity markers. Representative sequence-verified cDNA clones (Incyte Genomics,

Palo Alto, CA, USA) were queried by BlastN sequence comparison against other cDNA sequences sharing the same class of protein functional annotation. Clones sharing greater than 50% sequence identity respectively by BlastN were then selected for hybridization experiments. These clones were further scored for sequence homology using a pairwise sequence alignment algorithm, LALIGN (8). Corresponding GenBank® accession nos. were determined by matching the 5′ sequence read of each clone against the top hit in GenBank 117 (BlastN). Each clone contained the full-length coding insert, with the exception of the CYP family. The CYP family included examples of alternatively spliced genes. Twelve partial insert clones represented eight CYP homologs, three of which comprised splice variants.

To investigate hybridization cross-reactivity, an individual cDNA clone was selected from each gene family and designated as parent. This clone was used to generate a probe sequence for comparison against each cDNA clone sequence in the family. Pairwise homology scores were then calculated using a Smith-Waterman alignment tool, LALIGN (8). We decided to use this algorithm because it provides scores that represent both percent sequence identity and length of sequence alignment, and we hypothesized that such scores might provide a better prediction of cross-hybridization than sequence identity scoring alone. The reported alignment scores all exhibited greater than 50% sequence identity.

Preparation of PCR Targets, RNA Transcripts, and Fluorescent cDNA Hybridization Probes

The procedure used to generate PCR targets, parent RNA transcripts, and fluorescent hybridization probes is summarized in Figure 1. Each cDNA clone was amplified by PCR using vector-specific primers to generate a dsDNA target to be arrayed. To obtain RNA transcripts for the preparation of hybridization probes, designated targets were further amplified by PCR using gene-specific primers containing a T7-promoter sequence (5′-NNNNNN-TAATACGACTCACTATAGGGAG-3′) attached to the forward primer and a poly-dT_{30} sequence attached to the reverse primer, respectively. The T7 PCR templates were used to generate cRNA transcripts by incubation with T7 RNA polymerase (MEGAscribe™ kit; Ambion, Austin, TX, USA). Each RNA transcript was quantitated by fluorescence spectroscopy using an RNA-specific dye (RiboGreen®; Molecular Probes, Eugene, OR, USA). The fluorescence measurements were run in triplicate at two different dilutions, and the values were applied to a seven-step standard curve for quantitation. The integrity of each transcript was confirmed by agarose gel electrophoresis.

For hybridization experiments, pooled cRNA transcripts (typically 100 pg each unless indicated otherwise) were fluorescently labeled by reverse transcription with cyanine-3 or cyanine-5 dyes (GEMbright™ microarray labeling kits; Incyte Genomics). The resulting fluorescently labeled cDNA probes were purified by size exclusion chromatography (TE-30 column; BD Biosciences Clontech, Palo Alto, CA, USA), ethanol precipitated, and then resuspended in 24 µL hybridization buffer (5× SSC, 0.2% SDS, 1 mM DTT).

Microarray Fabrication

The PCR targets were purified using gel filtration over Sephacryl-400™ (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The targets were then concentrated and resuspended in 2× SSC for arraying. To minimize spot-to-spot variations, all PCR targets were normalized to a concentration of 100 ng/µL. In addition, the target elements were printed in quadruplicate onto each array for signal averaging. Microarraying was performed in the Incyte Genomics microarray facility (80–100 µm spots at 180-µm spacing, center to center). After arraying, the slides were irradiated at 120 mJ in a UV Stratalinker® model 2200 instrument (Stratagene, La Jolla, CA, USA) and then rinsed in 0.2% SDS for 2 min, followed by three 1-min rinses in deionized water. Slides were then treated with 0.2% I-Block® reagent (Tropix, Bedford, MA, USA)

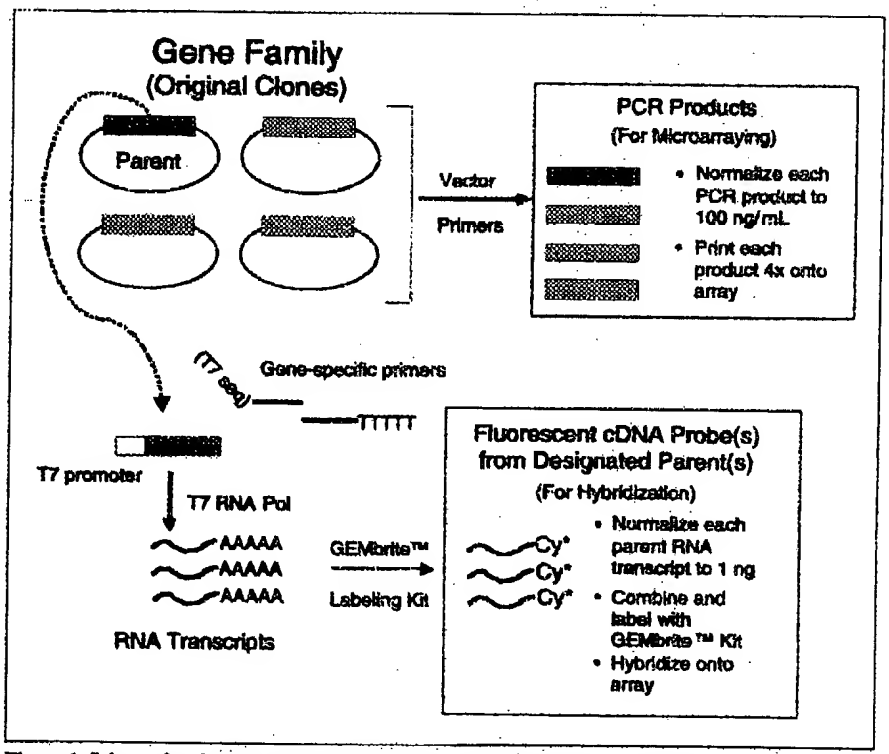

Figure 1. Scheme for the preparation PCR targets and fluorescent hybridization probes. Clones selected to represent each functional family were amplified by PCR using vector primers. One designated parent clone from each family was further amplified by PCR using chimeric gene-specific primers to produce RNA transcripts for use in the generation of fluorescent hybridization probes (see Materials and Methods).

in 1× Dulbecco's PBS (Invitrogen, Carlsbad, CA, USA) at 60°C for 30 min. The arrays were then rinsed again in 0.2% SDS and water as described above.

Hybridization and Data Analysis

Fluorescently labeled hybridization probes were applied to the microarrays under a 22 × 22 mm glass cover slip, placed in a sealed chamber to prevent evaporation, and then incubated at 60°C for 6 h. After hybridization, the microarrays were washed in 1× SSC/0.1% SDS/1 mM DTT at 45°C for 10 min and then in 0.1× SSC/0.2% SDS/1 mM DTT at 25°C for 3 min. The microarrays were then imaged using a GenePix™ dual-laser confocal scanner (Axon Instruments, Foster City, CA, USA) at 10 µm resolution. The scanned images were converted into 16-bits-per-pixel resolution, yielding a 65 536 count dynamic range. GEMtools™ software (Incyte Genomics) was used for image analysis. Four independent microarray hybridizations were conducted for each experiment. Target signals were corrected for local background and then averaged (four spots per array × number of arrays per experiment). Unless stated otherwise, four hybridization reactions were run per condition. Error bars shown in the figures are one standard deviation from the averaged signal values. Correlation coefficients between averaged hybridization signals and alignment parameters (clone length, overlap between the probe sequence and cDNA clone sequence, percent sequence identity, LALIGN score, etc.) were determined using Statistica® software (StatSoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

As stated in the Introduction, the purpose of this study was to measure hybridization cross-reactivity between cDNA targets sharing moderate to high sequence homology and to determine whether these cross-reactive signals could be correlated with their primary sequences (i.e., percent sequence identity, physical length, percent GC content, etc.). In particular, we considered that percent sequence identity might be a good predictor of cross-reactivity, based on reported cross-hybridization results on nylon membranes (15). Therefore, cDNA clones were selected from different functional gene families based on sequence identity scoring (i.e., the highest scoring clones available). In addition, one clone from each family was designated as a "parent" for hybridization comparisons based on the highest combined identity score.

We next considered whether the homology-scoring algorithm LALIGN could be used as a predictor of cross-reactivity between known sequences. In this case, we took into account the actual sequences present in the hybridization reaction. As described in the Materials and Methods section, a synthetic RNA transcript was generated from each designated parent using chimeric gene-specific primers. Whenever possible, these primers were designed near the ends of the first-round PCR products. However, this was not always practicable, so a number of the resulting transcripts span only a portion of the designated cDNA clone. These synthetic RNA transcript sequences were used for homology scoring by LALIGN against each cDNA clone of the family because they represent the actual templates used in the preparation of fluorescent hybridization probes. The respective homology scores and other sequence parameters are summarized in Table 1.

Hybridization Cross-Reactivity in the Serine Protease, Metalloprotease, Chemokine, and G-Protein Families

Hybridization probes generated from selected parent cDNA sequences were hybridized to a spotted cDNA microarray comprising all of the cDNA clones chosen for this study (see Materials and Methods). In the first hybridization experiment, replicate hybridizations were conducted using probe sequences from the serine protease, metalloprotease, chemokine, and G-protein families. The results from this experiment are summarized in Figure 2. Within each family, the targets corresponding to the designated parents were well discriminated from the other targets (cross-reactivities between 0.6% and 6%). As shown in Table 1, the highest cross-reactivities corresponded generally to the clones exhibiting the highest homology scores. However, despite similar homologies, the magni-

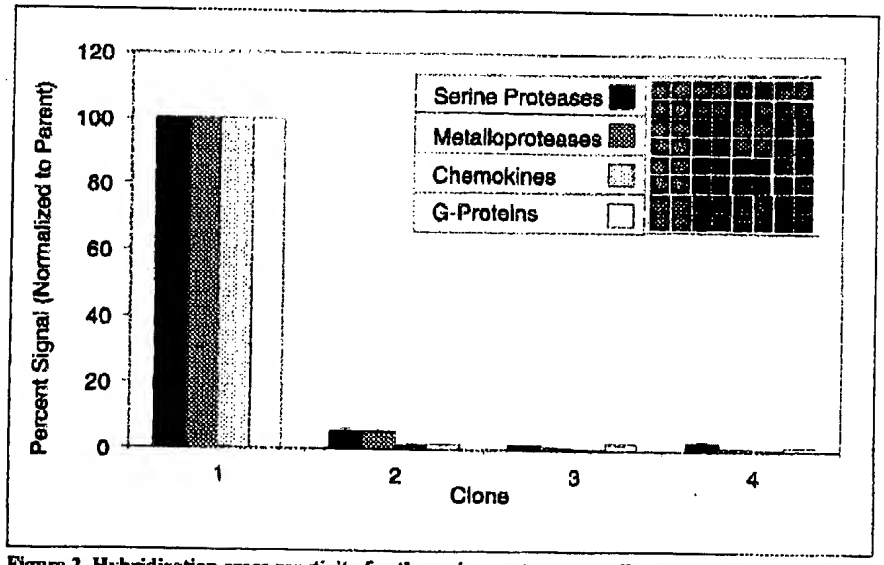

Figure 2. Hybridization cross-reactivity for the serine protease, metalloprotease, chemokine, and G-protein families. For these hybridization experiments, the probe reactions contained a pool of parent RNA transcripts (1 ng). This RNA concentration was selected to provide hybridization signals within the top third of the linear response curve for the scanner. The clones used to generate microarray targets for this experiment are summarized in Table 1. Inset: Hybridization signals shown as false color images (blue = bottom of the response curve < green < yellow < red < white = top of the response curve; note that not all colors may be represented in the figure insert).

Table 1. GenBank Accession Nos. and Alignment Scores for Spotted cDNA Clones Representing Homologous Gene Family Members[a]

Family	Clone	GenBank AccessionNo.	Transcript Overlap (bp)	Percent Identity	LALIGN Score	Percent Signal[b]
Serine Proteases	**1**	**g181189**	**816**	**100**	**3867**	**100**
	2	g4503136	464	65	721	5.5
	3	g35282	748	54	296	1.5
	4	g4504578	140	60	112	2.6
Metallo-proteases	**1**	**g4505204**	**1424**	**100**	**7063**	**100**
	2	g36632	1419	85	5079	5.2
	3	g903981	50	68	82	1.0
	4	g348020	139	55	77	0.9
Chemo-kines	**1**	**g1924937**	**497**	**100**	**2485**	**100**
	2	g4506840	373	68	713	2.9
	3	g4885586	405	64	603	0.9
	4	g3928270	181	80	572	0.6
G-Proteins	**1**	**g616866**	**164**	**100**	**820**	**100**
	2	g6912393	164	68	352	1.5
	3	g7022042	163	67	329	1.9
	4	g3329379	151	59	197	1.3
Threonine Proteases	**1**	**g4506204**	**243**	**100**	**1194**	**100**
	2	g565646	112	56	83	0.7
	3	g4506196	51	71	96	0.7
	3	**g4506196**	**533**	**100**	**2665**	**100**
	2	g565646	53	62	73	6.3
	1	g4506204	131	54	91	12.3
p450 (CYP)	**1**	**g181299**	**484**	**100**	**2420**	**100**
	2	g2618613	489	85	1638	56.9
	3	g2618613	500	82	1492	28.1
	4	g181343	477	86	1779	37.1
	5	g181327	476	80	1552	26.2
	6	g6470140	433	71	1019	8.1
	7	g181357	416	60	595	5.7
	8	g181299	106	100	530	33.6
	9	g6470140	110	76	316	4.4
	10	g181293	243	58	285	10.6
	11	g181269	55	60	80	6.2
	12	g6470140	39	67	66	6.0

[a]Bold, selected parent clone.

[b]Signal intensities as a percentage of the selected parent clone. For the CYP family, values are shown for the experiment summarized in Figure 3d that was hybridized at 60°C.

tudes of cross-hybridization differed between gene families. For example, in the chemokine family, an averaged cross-hybridization signal of 2.9% was observed between the parent (monocyte chemotactic protein-2, g1924937) and a second family member (monocyte chemoattractant protein-1, g4506840). The latter target exhibited 68% sequence identity with an LALIGN score of 713. In the serine protease family, clone g4503136 scored similarly (65% sequence identity with an LALIGN score of 721), yet gave a slightly higher cross-reactivity (5.5%). In the case of the metalloprotease family, comparably low cross-reactivity (5.2%) was observed for a clone exhibiting considerably higher homology (85% sequence identity over 99% of the designated parent probe sequence, with an LALIGN score of 5079).

Crossover Hybridization Experiment in the Threonine Protease Family

Hybridization probes from two clones of the threonine protease family (g4506204 and g4506196) were hybridized separately to the same cDNA microarray described above to determine whether alternative parent clone nominations could yield similar results.

In both assays, the non-parent targets were well discriminated from the selected parents (Table 1). However, the magnitudes of cross-hybridization were different in each case. Cross-reactivities measured for clone g4506204 were low (about 0.7%), whereas those measured for clone g4506196 were slightly higher (6.3% and 12.3%). This difference cannot be explained by the homology scores, which were similar, although the RNA transcripts generated from these two clones had different lengths (243 and 533 bp, respectively). Therefore, according to these results, homology scoring does not appear to be an exact predictor of cross-hybridization.

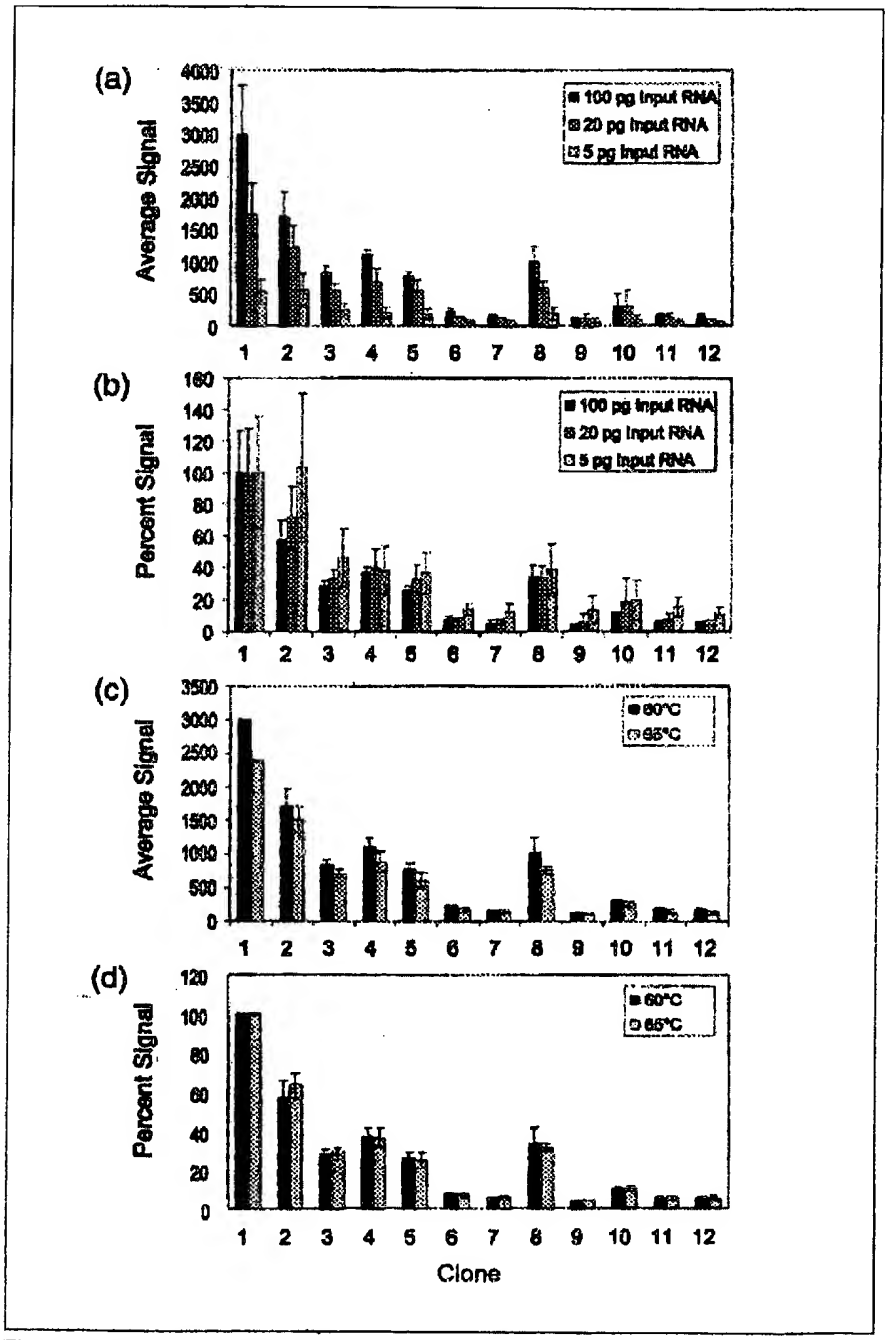

Figure 3. Hybridization cross-reactivity for the cytochrome P450 family. (a and b) Three separate sets of hybridization experiments are summarized using low (5 pg), medium (20 pg), and high (100 pg) input RNA transcript for probe generation ($n = 2$ per condition). (c and d) Hybridization results from 100 pg input RNA transcripts following hybridizations at standard (60°C) and elevated (65°C) temperatures ($n = 3$ per condition).

Cross-Hybridization in the CYP Family

Additional experiments were conducted with the CYP family to examine hybridization cross-reactivity over a range of input RNA concentrations, as would be expected from a complex biological sample, and also at different hybridization temperatures. Hybridization probes generated from the CYP parent clone (g181299) were hybridized to the same cDNA microarray described in the previous experiments. This family comprised a wider range of sequence homologies, including multiple examples of sequence identities greater than 80%. In several cases, the clones represented splice variants or different subclones of the same gene. One of these clones (clone 8) comprised a splice variant of the designated parent clone. The results from these experiments are summarized in Figure 3. The magnitudes of cross-reactive signals increased with input RNA concentrations (Figure 3a); however, the percent signal was relatively constant between these inputs (Figure 3b).

Raising the hybridization temperature from 60°C to 65°C resulted in slightly lower averaged signals (Figure 3c), but again the percent signals relative to the parent were similar (Figure 3d). These data suggest that cross-hybridization on microarrays is relatively constant and reproducible over the range of RNA inputs and temperatures investigated.

These cross-reactive signals showed a reasonable correspondence to the homology scores. Clones with less than 80% sequence identity (clones 6, 7, and 9–12) were well discriminated from the parent clone (cross-reactivity ranged between 4.4% and 10.6%). On the other hand, clones with 80%–86% sequence identity (clones 2–5) showed less discrimination (cross-reactivity > 25%). Additionally, one clone (clone 8) scored 100% sequence identity over about one-quarter of the parent transcript and showed about 33.6% cross-reactivity. This clone represents a splice

Table 2. Correlation of Cross-Hybridization Signals with Percent Sequence Identity and LALIGN Homology Scores Correlations of Cross-Reactivity Across families (All Data, I = 27)

Correlations of Cross-Reactivity across Families (n = 11)			
	Overlap	Identity	LALIGN
Percent Signal	0.19	**0.65**	0.34
Correlations of Cross-Reactivity for CYP Family Only n = 11)			
	Overlap	Identity	LALIGN
Percent Signal	0.52	**0.75**	**0.76**

Bolded values are significant at P<0.05.

variant of the gene corresponding to the parent clone.

Correlation of Hybridization Cross-Reactivities with Sequence Overlaps and Homology Scores

Statistical analysis of cross-reactive hybridization signals is summarized in Table 2. Across all families, only sequence identity showed significant correlation with signal intensity. Assuming that LALIGN might show a stronger correlation within a family (since sequence overlaps would be expected to vary widely between families), the analysis was repeated including only data for the p450 (CYP) family. Again, sequence identity showed the strongest correlation, although LALIGN also showed a significant correlation in this case. Taken together, these results indicate that hybridization cross-reactivity can best be predicted by sequence identity and that it is relatively independent of sequence overlap (at least above 100 bp, based on our data). It is interesting to note that the correlation score for sequence overlap increased in significance when the data for the CYP family was treated separately (Table 2). Five of the 11 clones in this family share greater than 80% sequence identity relative to the designated parent, including one splice variant (clone 8) that is 100% identical over a region about one-fifth of the parent transcript sequence. Therefore, the length of sequence overlap can have a greater effect on cross-hybridization in cases of high sequence homology, particularly in cases of sequences that represent alternatively spliced genes.

The results obtained from this study demonstrate that glass cDNA microarrays can generally distinguish hybridization between cDNA targets sharing less than 80% sequence identity. These results are in good agreement with previous data on nylon membranes where hybridization cross-reactivity was not observed until the alignments exceeded 77% sequence identity (15).

In the case of the CYP family data, substantial cross-reactivity was observed in two instances: the first case resulted from sequences exhibiting greater than 80% identity over of the majority of the probe sequence; the second case was for one sequence having 100% identity over about one-fifth of the probe sequence. These data indicate that higher levels of hybridization cross-reactivity can be expected in cases of high sequence identity over a majority of the sequence or exact identity over a portion of the sequence. Thus, splice variants are also predicted to show hybridization cross-reactivity, depending on the extent of sequence overlap with the clone represented on the array.

Application of Cross-Hybridization Results to Microarray Design and Gene Expression Analysis

Pairwise sequence alignment algorithms are widely used to elucidate conserved structural, functional, and evolutionary relationships among gene families (6,7). The data presented here demonstrate that sequence alignments and identity scoring can also be used to estimate hybridization cross-reactivity within gene families. When performing these calculations, it is important to consider the sequences that are actually arrayed. For example, clones representing a particular target gene may only contain a portion of the consensus gene

sequence that is available. Regions of the gene that are outside of the clone sequence should be excluded from these calculations. In the present study, the sequences of the cRNA transcripts generated from the selected parent clones were used for homology scoring by LALIGN because these were the templates that were used for probe generation. In the case of a biological sample, the full-length target sequence (if available) would be preferred.

With one exception (i.e., the 65°C hybridization temperature investigated with the CYP family), the conditions of the microarray experiments reported here were conducted according to the standard conditions of probe labeling, hybridization, and data analysis that are employed in our production process. In practice, hybridization experiments under different conditions (buffer, temperature, denaturants, etc.) have failed to yield improved discrimination (data not shown). However, it is possible that other stringency conditions not yet attempted could yield reduced cross-reactive signals. These studies are ongoing.

While the results presented here suggest that homologous regions of genes should be avoided when selecting clones for cDNA microarrays, they also indicate that cross-species hybridizations could be conducted on microarrays (i.e., where expressed gene sequences between the two species share 80% sequence identity or higher). This suggests that human cDNA microarrays could be used to analyze tissues from animal models of human disease. For example, a recent comparative analysis of orthologous genomic loci from human and mouse showed that genes could be identified in the latter based on conserved exon features (1). This predicts that expressed sequences in the human and the mouse will have sufficient homology for pathway analyses using cross-species microarray hybridizations. Indeed, preliminary investigations in our laboratory suggest that this is the case (P. Scott Eastman, unpublished results).

It is interesting to note that of the two gene families displaying greater than 80% sequence identity (metalloproteases and CYP) only the CYP family showed significant cross-hybridization from clones mapping to different genes. Therefore, as discussed earlier, pairwise sequence identity scoring is not an exact predictor of cross-hybridization but certainly needs to be considered when designing microarrays and interpreting the results.

ACKNOWLEDGMENTS

We thank Steven Daniel for technical contributions. We also thank Lyle Arnold, Rick Johnston, and P. Scott Eastman for many useful technical discussions and support, and Zora Modrusan and Mike Walker for critically reviewing this manuscript.

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Received 7 March 2001; accepted 1 August 2001.

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EXHIBIT E

Retrospective Study of *Chlamydia trachomatis* Using the Polymerase Chain Reaction on Archival Papanicolaou-Stained Cytologic Smears

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OBJECTIVE: To detect chlamydial DNA on archived Papanicolaou-stained (Pap) smears using the polymerase chain reaction (PCR) technique.

STUDY DESIGN: A PCR assay was designed to identify chlamydial DNA using consensus sequences unique to the genus Chlamydia *in the 16S rRNA gene. This assay produced a 109 base pair product containing a single Pvu II restriction site. One hundred cervicovaginal Pap smears from a teen clinic population were processed for DNA isolation and PCR. Amplifiable DNA was isolated from 93 of the 100 cases as determined by a human growth hormone gene. These specimens were subjected to chlamydial PCR.*

RESULTS: PCR analysis of the 93 samples yielded 6 that were positive for the chlamydial 16S rRNA sequence. The six positive chlamydial amplicons were purified and subjected to Pvu II restriction enzyme analysis to validate their identity. The analysis confirmed the identity of the products, as a single Pvu II restriction site resulted in 41 base pair and 68 base pair products, as predicted.

This method of detecting* Chlamydia *can be used on archival Papanicolaou-stained smears.

CONCLUSION: PCR testing for Chlamydia trachomatis *can be performed on DNA isolated from archival Pap smears. Using this methodology, 6.5% of young women in our teen clinic population were positive for chlamydial DNA.* (Acta Cytol 2001;45:985–989)

Keywords: *Chlamydia trachomatis*, Papanicolaou smear, polymerase chain reaction, retrospective analysis.

The state-of-the-art tests used for the detection of *Chlamydia trachomatis* are culture, DNA probe, enzyme immunoassay (EIA) and nucleic acid amplification.[1] Among these tests, cell culture has been

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Financial Disclosure: The authors have no connection to any companies or products mentioned in this article.

Received for publication July 21, 2000.

Accepted for publication January 25, 2001.

0001-5547/01/4506-0985/$19.00/0

considered the laboratory standard for the diagnosis of *C trachomatis* infection because of its 100% specificity and 80% sensitivity.[1,2] The disadvantages of the cell culture method are that it is technically difficult, costly and time consuming.[3,4] When compared to cell culture, the DNA probe method is less expensive and has a sensitivity of about 65%.[1] EIA is also less expensive and has a sensitivity of approximately 60%.[1] Nucleic acid amplification has a ≥90% sensitivity and specificity, although it is more expensive than DNA probe or EIA but less expensive than cell culture.[1]

The new commercial DNA amplification assays have exceeded the sensitivity of culture. Culture is now thought to have a sensitivity of 70–85%.[2] DNA amplification assays, such as polymerase chain reaction (PCR) and ligase chain reaction (LCR) are highly sensitive and specific when used on cervical specimens and urine samples.[2] The commercially available PCR test for *Chlamydia* (Amplicor®, Roche Biomedical, Palo Alto, California, U.S.A.) detects a highly conserved plasmid found in most *Chlamydia* species. This differs from the ribosomal gene target used in the present assay. The 16S rRNA gene was selected because several hundred copies of the gene are present in each organism. Theoretically, the higher copy number per organism of the 16S rRNA target sequences will increase the sensitivity of the PCR test when compared to plasmid targeted testing. Below we report the successful isolation of amplifiable DNA from archival Pap smears and detection of DNA sequences consistent with *C trachomatis* infection.

Materials and Methods

PCR Test Design for C trachomatis

The target 16S rRNA gene sequences that are unique to the genus *Chlamydia* were identified by the alignment of the *C trachomatis* 16S rRNA gene sequence with that of *Streptococcus agalactiae, Escherichia coli, Gardnerella vaginalis, Mycoplasma hominis, Neisseria gonorrhoeae, Treponema pallidum* and *Ureaplasma urealyticum*. The 16S rRNA gene sequences were obtained from the National Institute of Health (NIH) GenBank. Using a Clustal W program (Omiga 1.1, Oxford Group, Oxford, U.K.), an initial round of sequence alignment identified several candidate regions of the *C trachomatis* 16S rRNA gene for primer sequences. To be certain that all potential variations of the 16S rRNA sequence that may occur in *C trachomatis* would be detected, a second alignment was performed. In that alignment, 16S rRNA sequences of the following chlamydial species were incorporated: *trachomatis, pneumoniae, psittaci, felis* and *muris*, as well as *Lactobacillus vaginalis*. Three candidate regions of consensus sequence unique to the genus *Chlamydia* were identified as the targets of complementary oligonucleotide primers for PCR assay development. As a final test to validate that the consensus sequences were unique, they were input as separate Basic Local Alignment Search Tool (National Center for Biotechnology Informatics, NIH, Bethesda, Maryland, U.S.A.) searches into the NIH GenBank. Each consensus sequence identified only *Chlamydia* and *Chlamydophila* organisms within the database. The PCR primers were made for the three regions and tested on positive control DNA isolated from a commercial cat vaccine that contains *Herpesvirus felis, Feline parvovirus* (distemper), *Feline calicivirus* and *Chlamydia psittaci* (Eclipse IV, Solvay Corp., Piscataway, New Jersey, U.S.A.).

Study Population

Teen clinic Pap smear specimens that had been fixed in 95% ethanol were retrieved from the slide archives in the Department of Pathology, University of Virginia Medical Center, from the years of 1991 and 1992. Teen clinic specimens were chosen because they represent a population at high risk for *C trachomatis*. One hundred cases within normal limits encompassing approximately 200 slides were processed for DNA isolation and PCR. Patient history was not available for review, as the samples were coded and made anonymous.

Specimen Preparation for PCR

The Papanicolaou-stained slides were soaked in xylene for one week to remove the coverslips. The slides were then destained with acid alcohol and air dried.[5] Using a fresh razor blade for each specimen, the entire cellular sample was scraped into a labeled microfuge tube. The slides were then discarded.

DNA was isolated using the QIAmp DNeasy tissue kit (Qiagen, Valencia, California, U.S.A.) according to the manufacturer's specifications. All samples were heated to 95°C for five minutes and rapidly cooled and stored at −20°C.

PCR for the Detection of C trachomatis

The oligonucleotide primers used were:

CP505F	5'-GCG TAC CAG GTA AAG AAG CAC C
CP613R	5'-CCT TTC CGC CTA CAC GCC CT

The PCR master mix contained 0.5 µM of each primer, 1.5 mM $MgCl_2$, 2 units of Taq polymerase (Qiagen) and 1X Taq buffer; 5 µL (~ 100 ng) of DNA isolated from the sample specimen was added to the 50-µL PCR reaction. This was subjected to the following thermal cycler conditions: 94°C for 45 seconds, 65°C for 1 minute and 72°C for 1 minute for 35 cycles followed by one cycle of 72°C for 5 minutes. The reaction was analyzed with agarose gel electrophoresis.

Human Growth Hormone (HGH) Gene

HGH was used to determine if each sample contained amplifiable DNA. The oligonucleotide primer sequences used were:

HGH F 1671	5′-CCC TCT TTT TAG CAG TCA GG
HGH R 1860	5′-TCC AGC CTC TGC AAA GTG AA

This PCR assay produced an amplified product of 189 base pairs. The following thermal cycler conditions were used in the HGH assay: 94°C for 45 seconds, 59°C for 1 minute and 72°C for 1 minute for 35 cycles followed by one cycle of 72°C for 5 minutes. The reaction was analyzed with agarose gel electrophoresis.

Purification of PCR Product and Restriction Enzyme Analysis

PCR product from DNA samples positive for chlamydial 16S rRNA was purified for restriction enzyme analysis using the Qiaquick PCR Purification Kit (Qiagen). Sixteen microliters of purified PCR product was digested with 20 units of Pvu II restriction enzyme (NE Biolabs, Beverly, Massachusetts, U.S.A.) according to the manufacturer's recommendations and analyzed with high-resolution agarose gel electrophoresis.

Agarose Gel Electrophoresis

Chlamydia and HGH PCR reactions were analyzed using 2.5% agarose (Denville Scientific, Metuchen, New Jersey, U.S.A.) gels with 1X Tris Acetate EDTA buffer containing 0.5 µg/mL ethidium bromide run at 90 V for 30 minutes. The gels were visualized under UV irradiation and photographed.

High-resolution electrophoresis analysis of Pvu II restriction was performed using 3% Metaphor agarose (FMC Corporation, Rockland, Maine, U.S.A.) in 1X Tris Borate EDTA buffer with 0.5 µg/mL ethidium bromide solution run at 90 V for 45 minutes. The gel was visualized under UV irradiation and photographed.

Results

DNA was isolated from a commercial cat vaccine that contained *C psittaci* and subjected to chlamydial PCR. As predicted, a product was formed with a molecular size of approximately 109 base pairs when visualized with gel electrophoresis (Figure 1).

HGH assay demonstrated that 93 of 100 samples contained amplifiable DNA. Of the 93 specimens, 6 were positive for *Chlamydia* by subsequent PCR analysis (Figure 2).

The six positive chlamydial products were subjected to Pvu II restriction enzyme analysis and cleaved in a predictable fashion, identical to that of the *C psittaci* control. This restriction digest resulted in two fragments approximately 41 and 68 base pairs in molecular size (Figure 3).

The results indicated a 6.45% positive rate of infection in the study population. The HGH results suggested that up to a 7% false negative rate of detection would have occurred if this quality control assessment of DNA had not been performed.

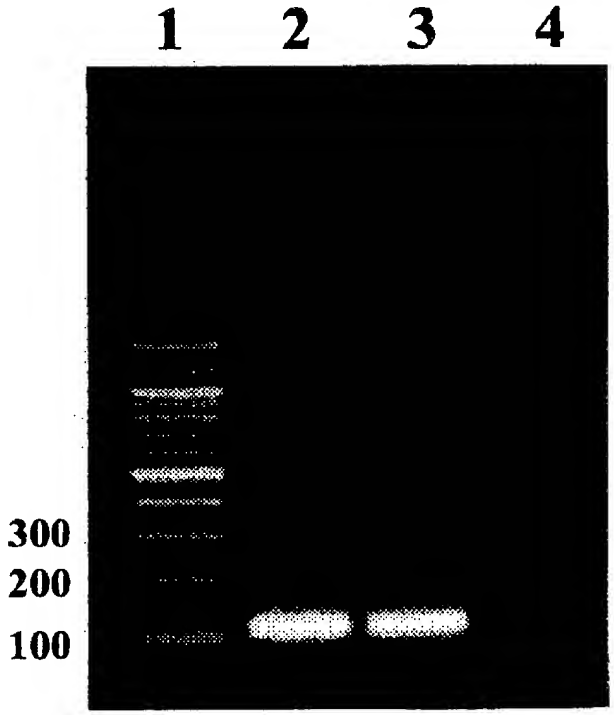

Figure 1 Photograph of a 2.5% agarose gel in 1X TAE buffer, 90 V, for 30 minutes. Lane 1 contains a 100–base pair molecular size ladder, lane 2 and 3 contain *Chlamydia* genus-specific PCR reactions with a *C psittaci* DNA template, and lane 4 contains mouse DNA (negative control). The PCR product is adjacent to the 100–base pair portion of the ladder.

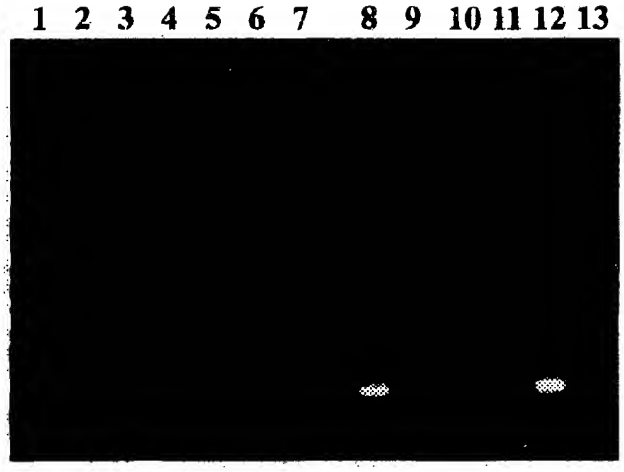

Figure 2 Photograph of a 2.5% agarose gel in 1X TAE buffer, 90 V, for 30 minutes. Lanes 1 through 10 contain *Chlamydia* genus-specific PCR reactions with Papanicolaou samples 1–10. Lane 11 is blank, lane 12 is *C psittaci* (+) control, and lane 13 is a water (–) control. Specimen 8 is positive for the *Chlamydia* sequence.

Discussion

C trachomatis is the most frequently reported sexually transmitted disease in the United States. It has been associated with a variety of clinical manifestations in both men and women.[6] In women, *C trachomatis* causes cervicitis, vaginitis, endometritis, salpingitis, pelvic inflammatory disease and infertility.[6] Symptoms, when present, may include a yellow vaginal discharge, pain during urination, lower abdominal pain, pain during sexual intercourse, spotting between menstrual periods, nausea and fever.[7] In men a chlamydial infection can lead to urethritis or epididymitis.[2] Infants born to infected women sometimes develop conjunctivitis and pneumonia.[2] Approximately 75% of infected women remain asymptomatic.[7] This silent venereal disease can cause serious, irreversible damage to the female genital tract, leading to infertility.[7] The cost of chlamydial illness in the United States is estimated to exceed 2.4 billion annually.[4] Screening methods leading to early detection of *C trachomatis* would decrease the costs of treatment and associated disease caused by untreated infections.[2] *C trachomatis*, when diagnosed, can be easily treated with antibiotic therapy.[7]

Sexually active adolescent women are at the highest risk for chlamydial infections.[8] The Centers for Disease Control and Prevention has recommended screening all sexually active females who are younger than 20 years of age for *C trachomatis* whenever they undergo a pelvic examination.[8] Reducing the high prevalence of chlamydial infection requires the awareness of health care providers so that they recognize chlamydial illness, screen asymptomatic patients, treat sexual partners and counsel all sexually active patients about the risks of sexually transmitted diseases.[4] The screening of

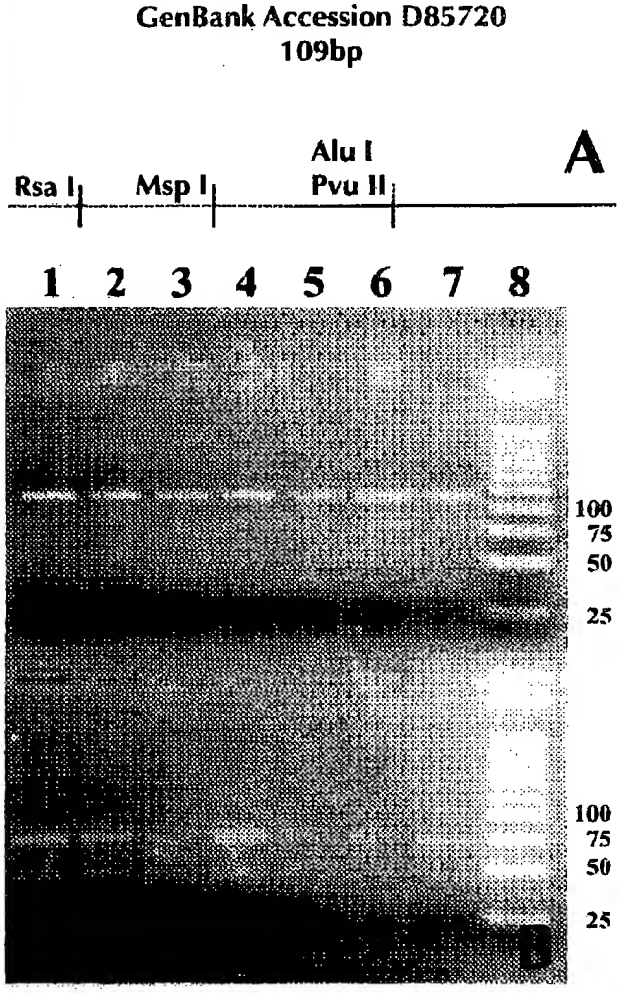

Figure 3 (A) Shown is a schematic representation of a restriction enzyme map of the 109–base pair fragment amplified by the *Chlamydia* genus-specific PCR. A Pvu II restriction site is located at nucleotide 41. (B) Photograph of a high-resolution 3% Metaphor agarose gel in 1X TBE buffer, 90 V, for 45 minutes. Lanes 1–6 contain the *Chlamydia* PCR 109–base pair product from samples 8, 11, 12, 19, 28 and 89. Lane 7 is the *C psittaci* (+) control, and lane 8 is a 25–base pair ladder. The upper row contains the purified, undigested PCR product, and the lower row contains Pvu II restriction digest products 41– and 68–base pair fragments.

women for chlamydial infections is a critical component of preventing chlamydial infections since many of these infections are asymptomatic and infections can persist for extended periods of time.[4]

Chlamydial PCR cervical tests are commercially available to laboratories testing for *Chlamydia*.[8] Urine testing of specimens as a method of detecting *C trachomatis* infection in women is currently under review by the Food and Drug Administration but have been approved for use in specimens from men. LCR, another DNA amplification chlamydial test, has been approved for both cervical and urine testing in women.[8] Even though these tests are available to clinical laboratories, they are not used routinely. Most physicians in clinics treat symptomatic patients for chlamydial infection. Some patients waiting for laboratory test results do not return for follow-up or treatment after their initial visit. Therefore, it is advantageous for physicians in clinics to treat patients with symptoms of *Chlamydia* and not wait for test results.

The Pap smear is an easy and inexpensive method for the diagnosis of several types of cervicovaginal infection, including human papillomavirus, herpes simplex, and *Trichomonas*, among other cytologic findings. Pap smears are not useful for the diagnosis of *Chlamydia*.[6] Recently, Digene Corporation (Gaithersburg, Maryland, U.S.A.) received Food and Drug Administration approval to market its Hybrid Capture® II Chlamydia/Gonorrhea Test for use in cervical specimens. This nucleic acid hybridization assay uses enzyme linked to a ribonucleic acid probe as the basis of signal amplification to identify the presence of *Chlamydia* and/or *Gonorrhoeae* in women with or without symptoms of infection. The effective amplification of a positive signal is 3,000-fold due to enzymatic activity of alkaline phosphatase, resulting in the production of a color product.

Our method of detecting *Chlamydia* in archival Papanicolaou-stained smears using a standard PCR method has an effective theoretical amplification of 10^6–10^9. This method would also be useful for specimens initially collected for the purpose of testing for the presence of *Chlamydia*. In addition, this method of detecting *Chlamydia* can be used on archival Papanicolaou-stained smears. Further studies are warranted to compare the sensitivity of our technique with that of other methods of *Chlamydia* detection currently accepted as clinically valid.

Acknowledgments

Ms. Feldman thanks her senior advisor, Dr. Robert L. McKown, Integrated Science and Technology Department, James Madison University, Harrisonburg, Virginia.

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EXHIBIT F

Validation of a Diagnostic Multiplex Polymerase Chain Reaction Assay for Infectious Posterior Uveitis

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Objective: To valide a multiplex polymerase chain reaction (PCR) assay capable of simultaneously screening vitreous biopsy specimens for a panel of common pathogens in posterior uveitis.

Methods: A multiplex PCR assay using novel primer sets for cytomegalovirus (CMV), herpes simplex virus (HSV), varicella zoster virus (VZV), and *Toxoplasma gondii* was developed. The sensitivity of the assay was determined for purified pathogen DNA. Twenty-one vitreous specimens from patients with posterior uveitis were tested by both multiplex and monoplex PCR.

Results: Fewer than 10 genomes of VZV and fewer than 100 genomes of HSV, CMV, and *T gondii* could be detected using the new primer sets. When used in multiplex, the assay lost less than 1 log of sensitivity. Monoplex PCR detected pathogen DNA in 18 of 21 patient samples; multiplex PCR detected pathogen DNA in 15 of the 18 samples positive by monoplex PCR. None of 10 negative control samples were positive for pathogen DNA.

Conclusions: Multiplex PCR has adequate sensitivity to simultaneously screen a substantial differential diagnosis for posterior uveitis in a single reaction, without loss of specificity. This assay may reduce the time and cost involved in PCR-based molecular diagnostics of infectious pathogens.

Clinical Relevance: Mutiplex PCR may allow rapid diagnosis of infectious posterior uveitis.

Arch Ophthalmol. 2001;119:1315-1322

THE MOST COMMON identifiable causes of posterior uveitis are infectious. In immunocompetent patients, *Toxoplasma gondii* is the most common infectious cause of posterior uveitis,[1,2] while in patients with acquired immunodeficiency syndrome, cytomegalovirus (CMV) is the major cause of retinitis.[3] Other relatively common causes of posterior uveitis are infectious as well. Varicella zoster virus (VZV) and herpes simplex virus (HSV) have both been implicated as causative agents in acute retinal necrosis syndrome and progressive outer retinal necrosis. Both of these diseases are associated with poor visual prognosis.[4,5]

Prompt diagnosis of posterior uveitis is vital for early and proper treatment. Treatment regimens for acute retinal necrosis syndrome, CMV retinitis, and *Toxoplasma* retinochoroiditis have minimal overlap, and the appropriate regimen can be initiated only once the correct diagnosis has been made. The diagnosis of infectious posterior uveitis is usually based on clinical presentation and appearance. However, in a subset of patients, media opacity or atypical appearance can necessitate additional testing to support a diagnosis. Historically, dilemmas in posterior uveitis have been analyzed by means of local antibody production (ie, the Witmer coefficient of normalized intraocular to serum antibody titers[6]) or direct viral cultures.[7-10] Although local antibody production has utility for determining a cause of some cases of posterior uveitis, including toxoplasmosis and acute retinal necrosis syndrome,[11] it is not useful for others, such as CMV retinitis. Viral cultures from the eye have poor recovery, and some organisms (such as *T gondii*) are not readily cultured. In recent years, the polymerase chain reaction (PCR) has been used in the diagnosis of posterior uveitis. The PCR can directly detect RNA or DNA of the causative microorganisms, with sensitivity and specificity often greater than that of culture. Polymerase chain reaction assays have been developed for CMV, HSV, VZV, and *T gondii*, and have shown clear utility in

From the Departments of Ophthalmology and Visual Sciences (Drs Dabil and Van Gelder and Mss Boley and Schmitz) and Molecular Biology and Pharmacology (Dr Van Gelder), Washington University School of Medicine, St Louis, Mo. *None of the authors has a financial interest in any of the technologies discussed in this article.*

MATERIALS AND METHODS

PRIMER DESIGN

In designing a novel multiplex PCR, we reasoned that compatible primers would share similar annealing characteristics, sequence complexity of the amplicon, and size of the amplicon. Previous investigators have shown that use of short PCR amplicons leads to more rapid and specific amplification. We designed a protocol for performing short tandem amplification of multiple pathogens (STAMP), with the goal of producing primer sets for individual pathogens that could be combined to function in multiplex reactions. To find such primers, we designed a short computer program to scan the genomes of VZV, HSV-1 and HSV-2, CMV, and *T gondii*, and selected primers found in coding regions (with the presumption that these sequences would be more likely to be conserved than noncoding DNA). These are referred to as the STAMP primers (**Table 1**).

The individual primers were designed such that each would be 20 base pairs (bp) long; would be 60% rich in guanine (G) and cytosine (C) (G+C); would produce approximately 100- to 300-bp-long amplicons, with a total G+C content of 50% and no stretch of 20 bp with greater than 70% G+C; and would not cause primer dimers and would not amplify homologous genomic sequences in human chromosomal DNA or other common pathogens. Results were confirmed by nested PCR of the multiplex product with internal primers. The nested primers were 20 bp long; approximately 50% G+C content; and at least 10 bp internal to the outside primers. All primers were designed by means of sequence databases at the National Center for Biotechnological Information with the Basic Local Alignment Search Tool family of programs. All primers were synthesized in 50-nmol quantities by IDT, Inc (Des Moines, Iowa). Primer sequences and their locations within the target genes are shown in Table 1 and **Table 2**.

PCR CONDITIONS

Purified pathogen DNA for CMV, HSV-1, *T gondii*, and VZV (Advanced Biotechnologies Inc, Columbia, Md) were used to optimize the sensitivity of each monoplex PCR reaction. The individual monoplex and multiplex PCR cycling conditions were extensively optimized for denaturation, annealing, and extension temperatures; magnesium chloride concentration; number of cycles; and concentration of primers for each pathogen.

For each monoplex reaction, 5 µL of sample (either purified pathogen DNA or patient vitreous sample) was combined with 5 µL of 10× PCR buffer (500mM potassium chloride; 100mM Tris hydrochloride [pH 9.0, at 25°C]; 1.0% Triton X-100); 5 µL of 25mM magnesium chloride; 1 µL of 0.2mM each dinucleotide triphosphates (dNTP), 5 pmol of each primer of HSV, *T gondii*, and VZV or 10 pmol of each CMV primer; and 0.25 U of recombinant Taq DNA polymerase (Promega, Madison, Wis) in a total volume of 50 µL. For multiplex PCR, 5 µL of DNA was combined with the same concentrations of 10× PCR buffer, magnesium chloride, dNTP, and recombinant Taq DNA polymerase as for monoplex. The same concentrations of all HSV, CMV,

making diagnoses in patients with diagnostic dilemmas.[9,12,14]

One impediment to the routine use of the PCR technique is the necessity of performing a separate PCR reaction for each pathogen in the differential diagnosis. The serial detection of individual pathogens is time consuming and may be prohibitively expensive if a large panel of potential pathogens are tested. Multiplex PCR is a technique in which PCR reactions for multiple pathogens are performed simultaneously, in a single reaction. Multiplex PCR has been applied to differential diagnoses in several infectious diseases[15,16] but has not been used in uveitis. In this article, we describe the design and validation of a novel multiplex PCR technique to diagnose infectious posterior uveitis.

RESULTS

MONOPLEX PCR SENSITIVITY OF STAMP PRIMERS

The sensitivities of the STAMP primers were tested in monoplex PCR (ie, with only 1 primer pair per reaction) against serial dilutions of purified pathogen DNA. As shown in **Figure 1** (left), the sensitivities of monoplex STAMP PCR were approximately 10 genomic copies for VZV, CMV, and *T gondii* (**Figure 2**C, left), and approximately 100 virus genomes for HSV. Since viral DNA amounts were calculated by the manufacturer on the basis of the number of virions used in the DNA preparation (not accounting for recovery of the DNA), these sensitivities should be considered relative, not absolute. To determine whether these sensitivities were comparable to those achieved by current PCR diagnostic techniques, the same pathogen DNA dilutions were tested with published primers from other laboratories (Figure 1, right, and Table 3). When used in monoplex, STAMP primers were equally or more sensitive than existing primer sets. Of note, the STAMP VZV primer was as sensitive in monoplex as the conventionally used nested PCR primer set. No comparison measurements for *T gondii* were performed, as the STAMP *T gondii* PCR is based on the highly repetitive *B1* gene and is similar to primer sets already in use.[14]

MULTIPLEX PCR SENSITIVITY OF STAMP PRIMERS

We next sought to determine the sensitivity of STAMP primers when all primers were combined in a multiplex PCR reaction. Reaction conditions were identical to those for monoplex PCR, except that all primer sets were included in a single reaction. No false-positive bands were produced when multiplex PCR was performed on purified pathogen DNA. Sensitivity comparisons of monoplex and multiplex PCR are shown in Figure 2. Multiplex PCR for VZV was as sensitive as the monoplex PCR on the purified VZV DNA. The sensitivities for CMV, HSV, and *T gondii* decreased less than 1 log unit.

VZV, and *T gondii* primers were then added in combination in a total volume of 50 µL. Samples were amplified in 200-µL thin-walled tubes in an automated thermocycler with heated lid (RoboCycler Gradient 96; Stratagene, La Jolla, Calif). Cycling conditions were as follows: an initial 3-minute denaturation at 94°C followed by 35 cycles of 30-second denaturation at 94°C, 30-second annealing at 52°C, and 30-second extension at 72°C.

The specificity of positive multiplex PCR results was confirmed by dividing the primary PCR product and performing individual confirmatory PCR with individual nested primer sets. Nested primers are shown in Table 2. One microliter of a 1:100 dilution of positive multiplex PCR product was combined with 5 µL of 10× PCR buffer (composition as above); 5 µL of 25mM magnesium chloride; 1 µL of 0.2mM each dNTP; 5 pmol of each nested primer of HSV, *T gondii*, or VZV, or 10 pmol of each CMV primer; and 0.25 U of recombinant Taq DNA polymerase in a total volume of 50 µL. Reaction conditions were the same as for the monoplex PCR. All nested reactions could be performed simultaneously on the gradient thermal cycler.

Monoplex sensitivities were compared with published protocols.[13,17,18] Primer sequences are shown in **Table 3**. For HSV detection, an initial denaturation at 94°C for 10 minutes was followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 64°C for 45 seconds, and extension at 72°C for 45 seconds. For VZV detection, after an initial denaturation at 94°C for 3 minutes, 35 PCR cycles of denaturation at 94°C for 30 seconds, annealing at 44°C for 30 seconds, and extension at 72°C for 40 seconds were performed. The VZV was detected by means of nested PCR, with an initial denaturation at 94°C for 3 minutes and 7 cycles at 94°C for 30 seconds and at 72°C for 40 seconds. The CMV was amplified with an initial 3-minute denaturation at 94°C, and 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds.

All amplified DNA was detected by agarose gel electrophoresis on 2% gels stained with ethidium bromide.

VITREOUS SAMPLES

Anonymous primary vitrectomy specimens were obtained from our own and other practices. We obtained 16 vitreous specimens from cases of posterior uveitis that had previously been shown by other laboratories to contain viral DNA.[12] Three vitreous specimens from patients with active toxoplasmosis and 2 vitreous specimens from patients with clinical acute retinal necrosis or progressive outer retinal necrosis syndrome were obtained at local institutions at the time of vitrectomy. Ten negative control vitreous samples were obtained at the time of vitrectomy from patients undergoing macular hole repair (4 patients), retinal detachment repair (3 patients), clearance of diabetic vitreous hemorrhage (2 patients), or submacular surgery for neovascular complex secondary to age-related macular degeneration (1 patient). Vitreous samples were immediately frozen at the time of acquisition and stored at −20°C or lower until assay. Vitreous specimens were thawed at room temperature, and PCR inhibitors were eliminated by boiling the samples for 15 minutes before assay.[12]

VALIDATION OF MULTIPLEX STAMP WITH VITREOUS BIOPSY SPECIMENS

We tested the STAMP monoplex PCR assay on 16 vitreous samples collected from cases of posterior uveitis previously shown to contain viral DNA by means of established primer sets. Frozen vitreous samples for CMV (10 cases), HSV (3 cases), and VZV (3 cases) were used. The monoplex STAMP PCR assay yielded CMV in 8 of 10 CMV samples, VZV in 3 of 3 VZV samples, and HSV in 2 of 3 HSV samples (**Table 4** and **Figure 3**).

Single, positive bands were produced after multiplex STAMP PCR of these same samples in 5 of 8 CMV cases, 3 of 3 VZV cases, and 2 of 2 HSV cases. Several methods for confirmation of positive multiplex bands were attempted, including Southern hybridization of specific probes to immobilized multiplex PCR products, reverse Southern hybridization of labeled multiplex PCR product to immobilized specific probes, and nested PCR of multiplex products for individual pathogens. We found the last technique to be most rapid (approximately 30 minutes) and specific. Multiplex PCR products were diluted by 1:100, split into individual reactions, and amplified with each nested primer set. All samples that were positive by the multiplex PCR were specifically positive with the nested primer set for the appropriate pathogen. In each case, the detected pathogen agreed with the pathogen detected by monoplex PCR (**Figure 4**). One of the 10 tested vitreous samples produced 2 positive bands on nested PCR, for VZV and for *T gondii*. As only VZV was detected by monoplex PCR, it is possible that the *T gondii* signal either was a contaminating false-positive finding or represented a very rare commensal organism. Although the patient sample was initially classified as VZV, no clinical data were available to determine which diagnosis was more consistent with the clinical presentation.

To determine specificity of multiplex PCR for posterior uveitis, vitreous samples from 10 patients undergoing vitrectomy for nonuveitic conditions were analyzed using multiplex and nested confirmatory PCR. None of the 10 samples produced visible products when tested with either multiplex PCR or on nested PCR of the multiplex products (data not shown). Positive control reactions run simultaneously showed sensitivities of at least 100 genomes for all pathogens tested, and less than 10 genomes for all pathogens following nested confirmatory testing.

Five additional patient samples from patients with active posterior uveitis were tested by multiplex PCR. These included 2 vitreous samples from patients with a clinical diagnosis of acute retinal necrosis syndrome or progressive outer retinal necrosis syndrome and 3 vitreous samples from patients with a clinical diagnosis of ocular toxoplasmosis. For the patients with herpetic retinitis, one of the samples was found to be positive for HSV and the other was positive for VZV with the monoplex PCR with the use of either STAMP or conventional primers. Although no band was seen after multiplex PCR with either sample, af-

Table 1. Oligonucleotide Primer Sets Used in STAMP*

Primer Set		Primer Positions	Product, bp
CMV		*HHV5* DNA Polymerase (*UL54*)	
Forward	CCT TTC CCT CGG CTT CTC AC	1377-1394	201
Reverse	GGA CAT CCC GCG TTG TTT CG	1558-1577	
HSV		*HSV* DNA Polymerase (*UL30*)	
Forward	GTG TGG GAC ATA GGC CAG AG	64 308-64 327	190
Reverse	GCG ACA TCC CCG CCT AGT AC	64 478-64 497	
Toxoplasma gondii		*B1* gene	
Forward	CCC GCT GGC AAA TAC AGG TG	488-507	155
Reverse	CGC AGT ACA CCA GGA GTT GG	623-642	
VZV		*HHV3 ORF59*	
Forward	CGG TTG GGT TGT CTT CTG TG	100 446-100 465	152
Reverse	GCG ACG AAC CGT AAG CGT GG	100 578-100 597	

*STAMP indicates short tandem amplification of multiple pathogens; bp, base pairs; CMV, cytomegalovirus; HSV, herpes simplex virus; VZV, varicella zoster virus; HHV, human herpesvirus; and ORF, open reading frame.

Table 2. Nested Oligonucleotide Primer Sets*

Primer Set		Primer Positions	Product, bp
CMV			
Forward	CGG GTT CGG TGG TTA TCG AC	1436-1455	142
Reverse	CCA AGA CTA ACT CGC CTA AC	1558-1577	
HSV			
Forward	GCA GCA AGA TAA AGG TGA AC	64 341-64 360	126
Reverse	CCT GAA GGA CAA GAA GAA GG	64 447-64 466	
Toxoplasma gondii			
Forward	CAG AAA AGC CAC CTA GTA TC	530-549	113
Reverse	CGC AGT ACA CCA GGA GTT GG	623-642	
VZV			
Forward	CGG TTG GGT TGT CTT CTG TG	100 496-100 515	102
Reverse	CTG TAC GGT TCT CGC ATA AC	100 578-100 597	

*CMV indicates cytomegalovirus; HSV, herpes simplex virus; VZV, varicella zoster virus; and bp, base pairs.

Table 3. Oligonucleotide Primers of Previously Established Individual PCR Methods*

Primer Set		Primer Positions	Product, bp
CMV		Major immediate early gene	
Forward	CCA CCC GTG GTG CCA GCT CC	1307-1326	162
Reverse	CCC GCT CCT CCT GAG CAC CC	1449-1468	
HSV		DNA polymerase gene	
Forward	ATC AAC TTC GAC TGG CCC TTC	1402-1422	179
Reverse	CCG TAC ATG TCG ATG TTC ACC	1560-1580	
VZV		*ORF62* and *ORF63*	
Set A			
Forward	GTT TTG TAC TCC GGG TTG	110 721-110 738	386
Reverse	TTA CAT CCG ATG GCG TAG	111 084-111 106	
Set B			
Forward	GCG CGT TGA GGA CAT CAA CCG TGT T	110 757-110 781	328
Reverse	CAT CGT CGC TAT CGT CTT CAC CAC	111 059-111 082	

*PCR indicates polymerase chain reaction; bp, base pairs; CMV, cytomegalovirus; HSV, herpes simplex virus; VZV, varicella zoster virus; and ORF, open reading frame.

ter the nested PCR of the (invisible) multiplex product, these vitreous samples were positive for HSV or VZV, respectively. One of the 3 vitreous samples from patients with presumed ocular toxoplasmosis was positive for *T gondii* by means of monoplex PCR. The nested PCR was positive for *T gondii* on all 3 samples (**Figure 5**).

COMMENT

The PCR is a powerful technique for detecting pathogen DNA or RNA as an indication of infection.[12,19,20] It is rapid, taking only a few hours to complete, and requires only a

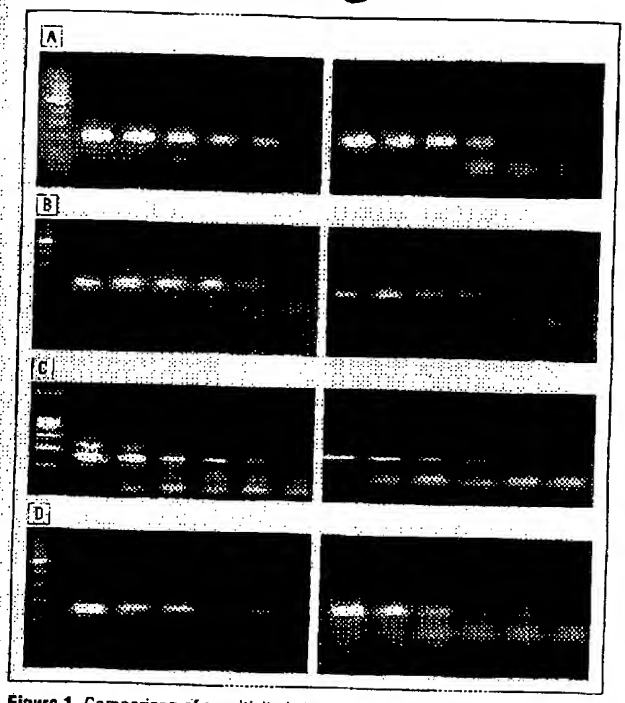

Figure 1. Comparison of sensitivity between monoplex STAMP primers (left column) and established polymerase chain reaction methods (right column) on purified pathogen DNA (A, cytomegalovirus; B, herpes simplex virus; C, varicella zoster virus). (STAMP indicates short tandem amplification of multiple pathogens. See the "Primer Design" subsection of the "Materials and Methods" section for an explanation of the STAMP protocol.) First lanes are the 100–base pair molecular size markers followed by 10-fold dilutions of the positive control viral DNA (beginning with DNA recovered from 10^5 virions). The last lane in each column is the no-DNA negative control.

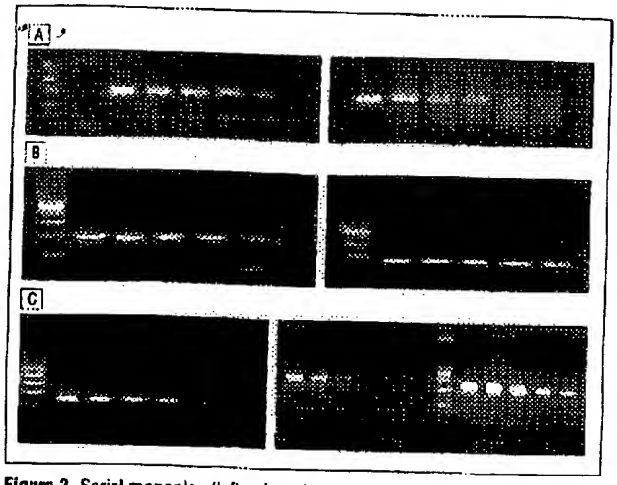

Figure 2. Serial monoplex (left column) vs multiplex (right column) detection with STAMP primers on purified pathogen DNA (A, cytomegalovirus; B, herpes simplex virus; C, *Toxoplasma gondii*; D, varicella zoster virus). (STAMP indicates short tandem amplification of multiple pathogens.) First lanes are the 100–base pair molecular size markers followed by 10-fold dilutions of the positive control pathogen DNA (beginning with DNA recovered from 10^5 pathogens). The last lane in each column is the no-DNA negative control.

Table 4. Summary of PCR Results*

Specimen	No. of Specimens	PCR Amplification, No. Positive		
		Monoplex	Multiplex	Nested
Frozen samples				
CMV	10	8	5	5
HSV	3	2	2	2
VZV	3	3	3	3
Fresh samples				
HSV	1	1	0	1
Toxoplasma gondii	3	1	1	3
VZV	1	1	0	1

*PCR indicates polymerase chain reaction; CMV, cytomegalovirus; HSV, herpes simplex virus; and VZV, varicella zoster virus.

few microliters of sample volume. The technique is extremely sensitive and specific; as we demonstrated in this study, sensitivities on the order of 10 to 100 pathogen DNA molecules can be routinely obtained. Knox et al[12] demonstrated the utility of PCR in establishing a diagnosis in cases of posterior uveitis that presented as a diagnostic dilemma because of media opacity, atypical appearance, or atypical response to treatment. This group detected a specific virus by PCR in 24 (65%) of 37 cases examined. The PCR diagnosis was consistent with the ultimate clinical course in each case. Knox et al did not perform PCR for toxoplasmosis in this study; as several of the cases in their study followed a clinical course consistent with *T gondii* infection, the yield of PCR diagnoses would likely have been even higher had this group been able to perform PCR for the parasite. Mitchell et al[21] similarly tested vitreous from 50 patients with retinitis and acquired immunodeficiency syndrome, determining a diagnosis in 47. Their PCR-based assays were similarly consistent with the clinical courses of these patients.

Although PCR is a rapid and useful diagnostic technique for detection of common posterior uveitis pathogens, it has not yet achieved widespread or routine use. Obstacles to the routine use of PCR include the relatively small number of laboratories performing the technique, the lack of standardization among laboratories, the lack of clinical experience in interpretation of results (particularly negative results), and the expense and time involved in performing this technique. One of the major hurdles in the performance of diagnostic PCR is the necessity of testing for individual pathogens serially. Because protocols for individual pathogens have been derived in independent laboratories, optimal buffer and cycling conditions rarely allow for simultaneous performance of assays. Serial testing becomes expensive and time-consuming for a large differential diagnosis. Sample may also become limiting if a large number of PCR reactions need to be performed. The multiplex PCR was initially described in 1988.[22] This technique involves detecting multiple targets simultaneously, in a single reaction. Multiplex PCR has had limited application to ocular or systemic infectious diseases.[15,16] For ocular disease, Jackson et al[16] reported a multiplex and degenerate PCR for the detection of adenovirus (subgenera B, C, D) and HSV from conjunctival swabs. This group was able to detect adenoviral DNA in 5 of 6 specimens culture-positive for adenovirus and HSV DNA in 5 of 6 specimens culture-positive for HSV.

Further efforts to apply multiplex PCR to diagnostic applications have been limited by declining sensitivity and

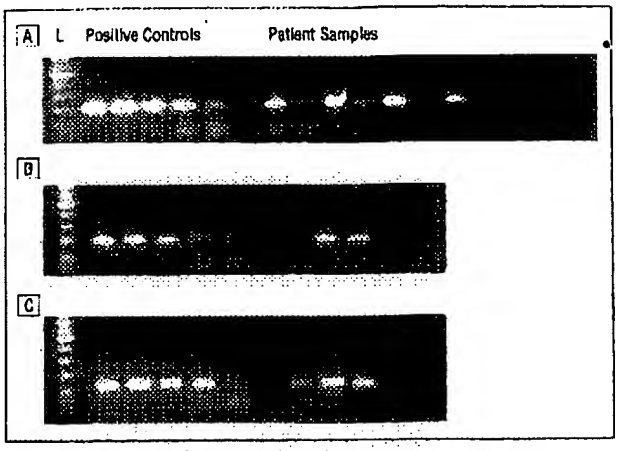

Figure 3. Monoplex detection of frozen patient samples with STAMP primers. (STAMP indicates short tandem amplification of multiple pathogens.) First lanes are the 100–base pair molecular size markers followed by 10^5 to 10^1 dilutions of the positive controls, 10 cytomegalovirus-positive samples (A) (eighth patient who was cytomegalovirus positive with the short tandem amplification of multiple pathogens is not shown), 3 herpes simplex virus–positive samples (B), and 3 varicella zoster virus–positive samples (C). L indicates DNA marker ladder. The last lane in each row is the no-DNA negative control.

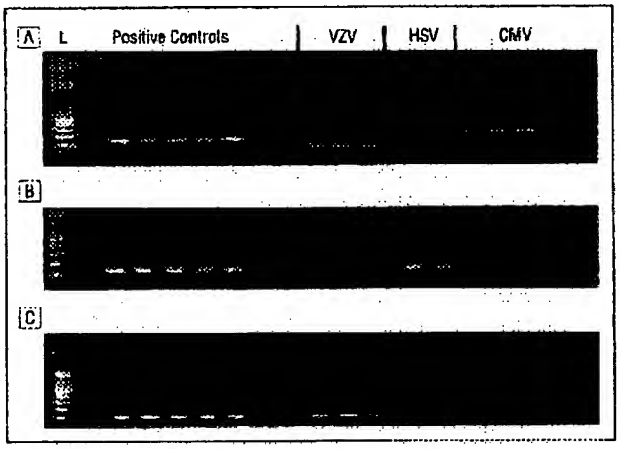

Figure 4. Nested polymerase chain reaction (PCR) on patient samples with each nested primer set (nested primer sets: A, cytomegalovirus [CMV]; B, herpes simplex virus [HSV]; C, varicella zoster virus [VZV]). First lanes are the 100–base pair molecular size marker followed by 10-fold serial dilutions of a 1:100 dilution of "first-round" PCR product generated from purified pathogen DNA (extracted from 10^5 to 10^1 pathogens). Row A is CMV-positive controls; row B, HSV-positive controls; and row C, VZV-positive controls. A 1:100 dilution of each postive PCR product from Figure 3 was tested with each nested primer pair. A fifth CMV-positive sample was also observed (not shown). All nested PCR products were also tested with *Toxoplasma gondii* primers; one VZV-positive sample was also positive for *T gondii* (not shown). L indicates DNA marker ladder. The last lane in each row is the negative control.

specificity with increasing complexity in the primer mix. As the number of primer pairs increases, the complexity of the DNA in the annealing reactions also increases, which decreases sensitivity. The likelihood of primer-dimer pairs also increases, causing nonspecific amplification and decreasing specificity. In designing a multiplex PCR for posterior uveitis, we sought to establish a generalizable method for generating primer sets and reaction conditions that would provide high sensitivity and ready compatibility with other primer sets. To that end, we wrote a short computer searching algorithm to find compatible amplicons.

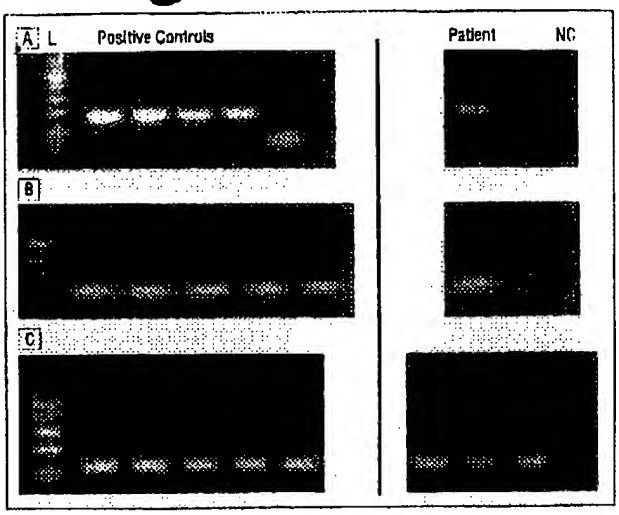

Figure 5. Nested polymerase chain reaction (PCR) on freshly acquired vitreous specimens. First lanes are the 100–base pair molecular size markers followed by serial 10-fold dilutions of the respective purified pathogen DNA (10^5 to 10^1) and 1:100 dilutions of primary multiplex polymerase chain reaction product from vitreous biopsy specimens of 1 patient positive for herpes simplex virus (A), 1 patient positive for varicella zoster virus (B), and 3 patients positive for *Toxoplasma gondii* (C). In each case, the PCR diagnosis was consistent with the clinical diagnosis. No patient was positive for more than 1 pathogen. L indicates DNA marker ladder. The last lane in each row is the no-DNA negative control (NC).

Each primer was to have a fixed length (20 bp), have a fixed (G+C)/(A+T) composition (60%), and flank a small intervening sequence (100-250 bp) with comparable (G+C)/(A+T) composition. We were able to find such sequences in all of the viral and protozoal genomes searched. Final choice of primer sequences was directed by location in the genome; coding sequences were favored over noncoding sequences. Genes with known mutational hotspots (ie, *UL97* of CMV[23]) were avoided. Using these criteria, we developed new primer sets for each of the major pathogens of posterior uveitis. When used in monoplex, these primer sets showed sensitivity equal to or greater than that of established primer conditions, averaging between 10 and 100 genomes in sensitivity. Reliable detection of less than 10 genomes by any technique is problematic, as serial viral DNA dilutions become subject to Poisson distribution variability. The primers were compatible in multiplex. Sensitivity of the multiplex assay was approximately 5- to 10-fold lower than that for monoplex. This is probably because of the greater total complexity of nucleic acids introduced by the additional primer sequences. It is possible that further optimization of the multiplex assay could yield improved sensitivity. For samples with limited volume, however, there is minimal loss of sensitivity for the multiplex assay when compared with monoplex. Given a 5-μL sample, for example, one could split the sample into four 1.25-μL samples and test each individually, or test the 5 μL in a single multiplex reaction. Because of the 4-fold dilution of sample in the monoplex reactions, the final sensitivities of each technique would be nearly equivalent. Although limiting sample volume is rarely limiting for vitreous or aqueous biopsy, it may be limiting for conjunctival swabs or fine-needle aspiration applications of PCR diagnostics.

Our primer sets were able to detect the appropriate pathogen DNA in 10 of 13 frozen archival samples and

5 of 5 freshly obtained samples. Most of the detection failures were from the CMV group. Several possibilities exist for the low recovery on these samples. Clinical isolates of CMV have been reported to exhibit a large degree of genomic polymorphism. For this reason, McCann and associates[13] used 2 different primer sets for CMV to test their samples. Our initial attempt at STAMP primers for CMV showed a sensitivity equal to that of existing primer sets on reference strain AD169, but was unable to detect CMV from a number of patient samples detectable by conventional primer sets. These primers may have been from a polymorphic site and thus did not amplify any product in the PCR-negative CMV samples. We did not have enough vitreous sample in these cases to repeat the PCR using established primers. It is also possible that the DNA specimens from the outside laboratory had undergone DNA degradation, as these samples had been freeze-thawed multiple times and were in some cases many years old (Todd Margolis, MD, PhD, oral communication, April 27, 2000).

The STAMP technique yielded a single apparent false-positive result, as 1 patient with a presumptive diagnosis of VZV-caused retinitis was also positive for *T gondii*. We believe this represents detection of actual pathogen DNA, as none of 10 tested nonuveitic samples were positive for any pathogen. Although early PCR assays for *T gondii* had relatively low sensitivity (approximately 30%),[2,24] more recent primer sets using highly repetitive *T gondii* genes such as the *B1* gene have yielded sensitivities from vitreous samples in the 60% to 70% range.[14] Recent primer sets have had sensitivity approaching 1 tachyzoite.[25] The prevalence of anti–*T gondii* antibodies in healthy adults in the United States is 40% or even higher,[26] and *T gondii* cysts have been isolated from clinically normal-appearing retinal sites.[27] Our false-positive result may represent a remote latent ocular *T gondii* infection, or it may have resulted from parasitemia from a nonocular site due to breakdown of the blood vitreous barrier, caused by the ocular VZV infection. In our use of PCR we have encountered several other apparent false-positive results for herpesvirus families. In particular, in cases with dense vitritis, a weak CMV-positive signal can be seen that may represent episomal virus latent in white blood cells.[28] Short et al[17] similarly found false-positive results for VZV when their nested PCR assay was fully optimized, possibly because of detection of rare copies of latent virus (perhaps even in the corneal and scleral nerves sampled during specimen acquisition). Performing a dilution series of positive control samples may help distinguish commensal from pathogenic infection for some infectious uveitides. In the present series of patients, for example, all patients with VZV-caused disease had semiquantitative PCR signals comparable to approximately 1000 viral genomes per 5 µL of vitreous. Commensal or carryover contamination would be expected to have lower viral loads.

Although the STAMP multiplex PCR technique is presently useful for diagnosis of posterior uveitis, the true utility of the technique will likely emerge as the differential diagnosis for PCR-detectable organisms grows. The PCR detection of less common causes of posterior uveitis, including Lyme disease,[29,30] syphilis,[31] mycobacteria,[32,33] lymphoma,[34,35] and even Whipple bacillus,[36] has now been reported. Serial examination for all of these diagnoses would tax available sample volumes and would likely be prohibitive in terms of time and expense. Suites of STAMP primers to evaluate classes of infectious posterior uveitis could be synthesized. Similarly, PCR-based diagnosis of infectious endophthalmitis[37-41] requires the ability to detect any of a large number of potentially causative microorganisms. Although the use of "universal" ribosomal DNA primers allows detection of the presence of bacteria,[39,41] actual diagnosis requires precise typing. This is presently performed by sequencing, hybridizing, or restriction digest fingerprinting the universal ribosomal DNA product, but it could be performed with greater speed and high specificity through a STAMP-based multiplex PCR with the use of nested primer sets. Other future uses of multiplex PCR include the rapid diagnosis of infectious conjunctivitis (where multiple strain types of pathogens make serial PCR difficult), diagnosis of delayed-onset endophthalmitis,[39] and multiplex strain typing of pathogens with variable antibiotic responses, such as CMV[23] and *T gondii*.[42,43]

Accepted for publication March 8, 2001.

Dr Van Gelder is supported by the Research to Prevent Blindness Career Development Award (New York, NY), the Becker Clinician-Scientist Award of the Horncrest Foundation (Ossining, NY), and the Thomas D. and Ruth Byers Heed Fellowship (Cleveland, Ohio). This work was supported by an unrestricted departmental grant from Research to Prevent Blindness, and a departmental core grant from the National Eye Institute, Bethesda, Md.

We thank Todd P. Margolis, MD, PhD, and Daniel F. Martin, MD, for kindly providing the reference vitreous samples for this study; Travis Meredith, MD, Henry Kaplan, MD, and Levent Akduman, MD, for providing additional patient samples; and Henry Kaplan, MD, and Michael Kass, MD, for support and encouragement.

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